BIOPROCESSING OF MICROALGAE *C. REINHARDTII* FOR PRODUCTION AND PURIFICATION OF SINGLE CHAIN ANTIBODY FRAGMENT

A Thesis

by

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ABSTRACT

The eukaryotic green alga, *Chlamydomonas reinhardtii*, is a unique expression platform that can efficiently express complex therapeutic proteins. However, demonstrating that therapeutic molecules can be produced in quantifiable levels is essential to establish the potential of the *C. reinhardtii* expression system. Thus, the objective of the first part of investigation (Chapter II) was to determine the process conditions that could maximize *C. reinhardtii* biomass accumulation and induced-production of the two recombinant proteins, a single chain fragment antibody molecule (αCD22 scFv) and malaria vaccine antigen (Pfs25), produced in the chloroplast of *C. reinhardtii*. To achieve a higher production of recombinant proteins, cultivation variables of *C. reinhardtii*, such as mixing, light-induction time and intensity, nutrient depletion and culture age, were investigated and optimized. The optimal light-induction time was 24 h at a light intensity of 300 μmol m^{-2} s^{-1}. Replacement of the culture media in the late exponential growth with fresh media was beneficial to the accumulation of recombinant proteins. Optimization led to increases in the accumulation of recombinant proteins by six-fold and the recombinant protein fraction in the extracted soluble protein by two-fold.

The pharmaceutical applications of these molecules are limited because variables affecting downstream processes are not well understood; consequently the potential value of the product is being masked for the viability with regards to economics. Thus, the objective of second part of the investigation (Chapter III) was to examine extraction and pretreatment methods to maximize the recovery of αCD22 scFv from *C. reinhardtii* extract, while minimizing the amount of impurities recovered. The optimal extraction was in 50 mM Tris buffer with 400 mM NaCl and 0.5% Tween at pH 8. Different pretreatment methods, i.e., ammonium sulfate precipitation, acidic precipitation and polymer (chitosan) precipitation were evaluated based on their ability to reduce impurities and maximize recovery of αCD22 scFv from algal extract. All the pretreatments tested on cell-free extracts were effective in reducing the amount of impurities and turbidity, with no loss in yield of αCD22 scFv. However, in case of cell lysates, a significant loss (~30%) of αCD22 scFv was observed with all three pretreatments. Chitosan precipitation, in particular is a promising method, which significantly reduced all the impurities, including DNA and turbidity.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

1.1. Background

Protein therapeutics are rapidly growing market with products in most branches of medicine. These protein therapeutics include vaccines, immunotoxins, single chain antibody fragments, cytokines which have advanced to the cusp of clinical success [1]. They are currently produced by either mammalian cell culture which is expensive because of complex nutritional and growth requirements and specialized bioreactor design, or in *E. coli* which is inefficient in expressing properly folded, complex proteins as soluble molecules. Other alternative systems like yeast, plants and insect cells also have their share of limitations, which restrict their use commercially [2-4]

With simple growth requirements, rapid growth, scalable production, eukaryotic alga, *Chlamydomonas reinhardtii* offers an attractive alternative for production of therapeutic recombinant proteins. Our collaborators have already demonstrated that *C. reinhardtii* is able to produce complex, unique large dimeric functional immunotoxin, single chain antibody fragment, malaria vaccine proteins that cannot be efficiently expressed in and purified from other heterologous systems [2, 5, 6]. However, these unique therapeutic molecules have high-impact potential provided that *C. reinhardtii* is established as a viable production system from which the recombinant proteins can be produced and purified at a competitive cost on a commercial scale.

The thesis addresses broadly two different bioprocess technology challenges: (i) increasing production of recombinant proteins αCD22 scFv and Pfs25 in *C. reinhardtii* chloroplast by optimizing cultivation and light induction variables (upstream processing) (Chapter II) and (ii) maximizing recovery of αCD22 scFv in algae extract (Chapter III). The expression cassette designed by our collaborator to replace endogenous psbA gene with the transgenes regulated by endogenous psbA promoter provides unique opportunity for decoupling biomass production and protein accumulation. The overall goal of the project was to identify and address the various obstacles in establishing microalgae as a platform for therapeutic recombinant proteins.

1.2. Literature Review

1.2.1. Use of microalgae as a therapeutic production platform

Around 130 protein therapeutics have been approved by US Food and Drug Administration (FDA) in a span of over 25 years, which has led to successful treatment of diseases like diabetes, hepatitis, etc. These therapeutics include cytokines, vaccines, antibody based drugs, enzymes, etc. These therapeutics are produced in a number of heterologous protein expression systems. These systems have their distinct pros and cons with respect to yield of the protein, operation cost and ease of manipulation [7].

Cultured transgenic mammalian cells are currently the most popular expression system for production of complex mammalian proteins and monoclonal antibodies (mAbs). However, it has its share of shortcomings like complex nutritional and growth

requirements, need for specialized bioreactors, expensive operation, and, with exception of monoclonal antibodies, low expression titers [8]. While bacterial systems like *E. coli* are efficient in producing large quantities of recombinant protein economically, they have limitations with respect to production of properly folded complex proteins as soluble molecules with properly formed disulfide bonds [9]. Yeast expression system is also being evaluated but there is variability in their N-linked and O-linked glycosylation machinery compared to the machinery found in mammalian cells which frequently leads to proteins that are not suitable for use as human therapeutics [10]. Transgenic plants like tobacco are also currently being investigated as a therapeutic production platform as they are free from human pathogens, and capable of post- translational modifications. However, the long development time for transgenic plants [11, 12] along with regulatory uncertainty [13] and purification challenges are potential disadvantages that are being addressed [14]. There have been advancement and improvement in developing these traditional expression systems. However, these systems are not ideal for expression and production of certain complex therapeutic proteins such as cytokines and eukaryotic toxins which require disulfide bond formation (limits use of *E.coli* cells) and are toxic to eukaryotic translational machinery(limits use of CHO cells) [15]. The development of algae as a platform for therapeutic protein production provides an opportunity to address the challenges faced by established expression systems.

Surprisingly, there have been few reports on the generation of transgenic algae for the expression of recombinant proteins [4], even though green algae have served as a model organism for understanding everything from the mechanisms of light and nutrient

regulated gene expression to the assembly and function of the photosynthetic apparatus. Recent studies have demonstrated the potential of microalgae as a factory of recombinant protein production; i.e., 1) therapeutic proteins, which are difficult to produce and 2) industrial proteins as secreted high-value products during cultivation of microalgae [4, 6, 16]. Generally Recognized as Safe (GRAS) eukaryotic algae offer several advantages over other systems: (i) significant reduction in cost, (ii) length of time required to grow is relatively quick (doubling time 8-12 hours) [17, 18], (iii) it is easily grown in a full containment and is scalable, (iv) not susceptible to viral or prion contamination that can harm humans, as is always a concern with animal cell culture, (v) possess the chaperones and cellular machinery required to fold complex human proteins that bacteria and yeast may not be able to process properly, (vi) microalgae *Chlamydomonas reinhardtii* can be grown photosynthetically as well as non-photosynthetically with acetate as carbon source, and (vii) unlike transgenic plants, uniform cellular population of algae can provide an opportunity for robust purification with easier scale up and minimized product losses. All three genomes (chloroplast, mitochondrial, and nuclear) can be transformed in *Chlamydomonas*, and each has distinct transcriptional, translational, and post-translational properties that make them distinct. Chloroplast offers some unique attributes, i.e. (i) correctly folds and assemble complex mammalian proteins due to presence of chaperons and ability to form disulfide bonds, (ii) in algae that grow non-photosynthetically, the chloroplast can provide a protected intracellular space that is non-essential to cell growth, (iii) tools to genetically manipulate the chloroplast and over-express proteins already exist, (iv) accumulation of

higher level of recombinant proteins in chloroplast, mainly because there are minimum gene silencing mechanisms in plastids, (v) chloroplasts can be transformed with multiple genes in a single event, due to the availability of multiple insertion sites, as well as an ability to process polycistronic transcripts, allowing an entire gene cassette to be regulated by a single promoter, and (vi) chloroplast allows toxins that target the eukaryotic translational apparatus to be produced, where they are sequestered from the cytosol where they would otherwise be lethal to the host cell [2-5, 16, 19-21].

Eukaryotic green algae *C. reinhardtii* have been engineered to produce a wide range of recombinant proteins. Mayfield lab has demonstrated expression of ~40 proteins in *C. reinhardtii* chloroplast, all remained soluble with very low amounts of the recombinant proteins associated with insoluble cell fractions [4, 22]. These include a dimeric immunotoxin (αCD22-HCH23-ETA) for cancer treatment [6], cytokine (IL-17D) with tumor suppressing properties, malaria vaccine (Pfs 25) [5], and human monoclonal antibody against anthrax protective antigen 83 (PA83)[22] etc. Surzycki *et. al.* [23] analyzed factors affecting protein expression in *C. reinhardtii* chloroplast and listed the following main factors: correct codon optimization, protein toxicity, proteinase activity and transformation–associated genotypic modification [23]. Biolistic transformation methods have been used to introduce foreign DNA into the *C. reinhardtii* chloroplast. The DNA is inserted into specific sites in the chloroplast genome via homologous recombination and transformants are positively selected by using a kanamycin resistance gene that is delivered by the same plasmid bearing the recombinant gene of interest.

1.2.2. Single chain antibody fragment

Single chain antibody fragments (scFvs) are highly relevant in the development of diagnostics and therapeutics and account for 35% of antibody fragments in clinical trials [24-26]. An scFv fragment consists of the smallest functional antigen-binding domain of an antibody (~30 kDa), in which the variable heavy (VH) and variable light (VL) chains are joined together by a flexible peptide linker [27] (Figure 1). The linker (3.5 nm in length) usually have hydrophilic residues with stretches of Gly and Ser for flexibility [28, 29]. In comparison to full-length mAbs, scFv fragments have several advantages such as (i) improved pharmacokinetic properties due to better tissue penetration and rapid blood clearance, (ii) low immunogenicity due to absence of Fc region. Thus, scFv fragments are beneficial in radiotherapy, diagnostic and therapeutic applications. [30].

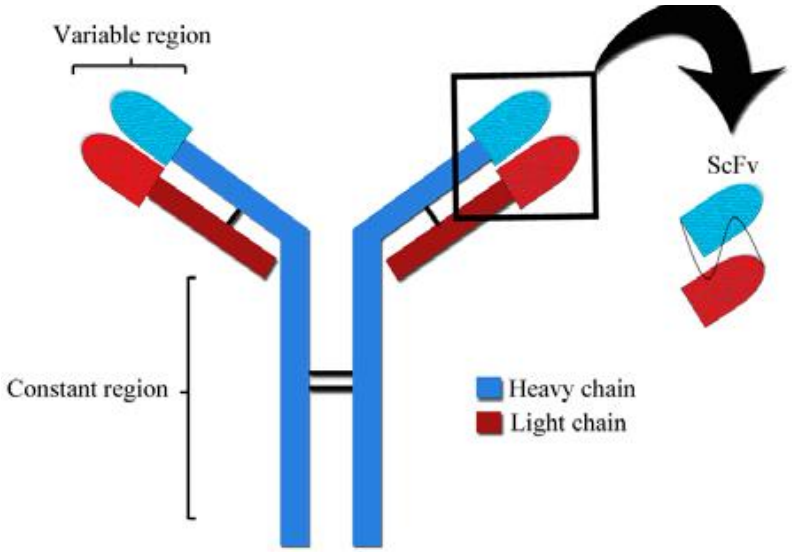

Figure 1. Schematic representation of full-length monoclonal antibodies (mAb) and single chain variable domain (Fv) fragments (scFv) fragments.

However, scFvs has several disadvantages too. For instance, its small size makes it unstable and prevents its accumulation at the target size, thus making it undesirable for therapeutic applications. This has been addressed by increasing their in vivo half-life and stability though various modifications, such as PEGylation. However, certain technical challenges need to be overcome to produce them cost-effectively in this format. Secondly, the lack of Fc-mediated cytotoxicity limits the therapeutic potential of scFvs. Thus, they are usually conjugated to drugs and toxins to be more effective in therapies [30].

scFvs fragments have a broad range of applications. They can be genetically manipulated and engineered to yield multivalent and multifunctional multimers (diabodies, triabodies and tetrabodies) that have higher avidity and lower blood clearance, without compromising tissue penetration abilities [31]. Moreover, scFv fragments can be further engineered so that they are linked to different moieties, such as drugs, toxins, radionuclides, quantum dots or liposomes [24, 32]. Other application of scFv includes its use as molecular tools to perturb protein functions in vivo [33, 34], delivery agents of radionuclides in diagnostic imaging [35] and as potential therapeutics for diseases like cancer, HIV and neurodegenerative diseases [36-38].

One of the applications of scFv is to construct specific immunotoxin molecules [39]. Recently, Mayfield group at UC San Diego has demonstrated the ability of eukaryotic algae *C. reinhardtii* chloroplast to produce and accumulate a unique full length immunotoxin as cancer therapeutic. Immunotxin has been produced by genetically fusing the single chain (scFv) antibody that recognizes the CD22 antigen

from B-cells leukemia and lymphomas with eukaryotic toxin (a truncated variant of *Pseudomonas aeruginosa* exotoxin A ETA). They demonstrated the production of single chain (scFv) antibody fragment, monomeric and larger dimeric active immunotoxins from algae which are capable of binding specifically to B cells displaying the CD22 molecule and causing them to undergo apoptosis [6].

1.2.3. Microalgae cultivation

At present, the most common procedure for cultivation of algae is photoautotrophic growth in open ponds. Photoautotrophic growth of algae in large open outdoor ponds has been used since the 1950s for production of single-cell protein, health food, and b-carotene [40, 41] and is one of the oldest industrial systems [42]. However, poor light penetration, costly and laborious harvesting, requirement of continuous and clean water supply and difficulty in mono cultivation of algae has restricted its use for production of pharmaceuticals and food ingredients. The numerous photobioreactors designed to overcome the disadvantage of the open pond system have been successful in achieving higher biomass productivity but its high cost in facility and operation and limiting light penetration on large scale leads to a lower economical viability than open pond [41]. Heterotrophic culture is a practical alternative for photoautotrophic culture. In heterotrophic cultures, organic carbons such as sugars and organic acids are used as carbon sources in the absence of light. Because the heterotrophic culture can be performed in conventional bioreactors, it is much easier to alter conditions to improve the yield of biomass and reduce the cost of microalgae biomass production [43]. The

heterotrophic growth of algae offers a promising alternative with fast algal growth, high production rate and convenient harvesting [44, 45]. Recently, heterotrophic microalgae culture has also been used on an industrial scale to produce biodiesel and polyunsaturated fatty acids. Under heterotrophic conditions, the growth rate, lipid content accumulation, ATP yield (mg of biomass generated/mg of ATP consumed), and N content are higher than under autotrophic conditions; however, the growth rate and amount of each of the products mentioned above are highly related to the algae species. Addition of oxygen under heterotrophic conditions is a key factor to maintain the growth rate and biomass production of high cell density cultures [41]. In mixotrophic growth, both respiratory and photosynthetic metabolism operates concurrently with assimilation of both CO_2 and organic carbon. This type of cultivation provides all the benefit of heterotrophic cultivation plus the ability of synthesize photosynthetic metabolites [45].

Heterotrophic cultures have several limitations including contamination and competition with other microorganism; inhibition of growth by excess organic substrate; and inability to produce light-induced metabolites [45]. Nonetheless, there are many practical advantages of heterotrophic growth of microalgae and hence it is gaining increasing interest for producing a wide variety of metabolites at all scales, from bench experiments to industrial scale [41, 46, 47].

Glucose [47, 48], Glycerol [49] and acetate are some common carbon source used for heterotrophic cultures of microalgae. Acetate (carried by coenzyme A) is generally oxidized metabolically through two pathways: (a) the glyoxylate cycle to form malate in glyoxysomes (specialized plastids in the glyoxylatecycle) and (b) through the

tricarboxylic acid cycle (TCA) to citrate in the mitochondria, which provides carbon skeletons, energy as ATP, and energy for reduction (NADH) [41]. In *C. reinhardtii* growing on acetate, the oxidative pentose phosphate pathway is also active, providing reducing power as NADPH for cytosol [50]. Additionally, mitochondrial and chloroplastic electron transport chains are active in these cells and have a close interaction through the glycolytic pathway [51].

1.2.4. Light induction for recombinant protein expression

It has been observed that codon optimization , promoter and regulatory mRNA untranslated region (UTR) choices are crucial factors in regulating transgene expression levels in *C. reinhardtii* chloroplast [52, 53]. Some of the first attempts to express recombinant proteins in the chloroplast of *C. reinhardtii* were under the control of promoters such as: *rbcL, psbD* and *atpA* [54-56]. But, the most successful promoter to date in algae is the psbA promoter in combination with the psbA UTRs [19, 23].

The Dl protein of photosystem II (PSIl) encoded by the psbA mRNA, is a reaction center protein that participates directly in photosynthetic charge separation and has been shown to be the most highly synthesized protein in illuminated photosynthetic cells [57]. In the *C. reinhardtii* strain studied, endogenous psbA gene coding for D1 protein, was replaced by the anti-CD22scFv (MT44) via direct homologous recombination of the psbA locus, by using a chloroplast expression cassette that contained anti-CD22scFv/immunotoxin and kanamycin (kan) resistance coding region. Since recombinant protein expression is controlled by the psbA promoter, transgene expression

is light inducible [5, 6, 57]. Light distribution in microalgae cultures is highly heterogeneous due to absorption and scattering by cells [58].

Light regulates translation of psbA regulated proteins in algae *C. reinhardtii* by modulating the binding of activator proteins to the 5' untranslated region of psbA messengerRNA in vivo. This is achieved by mechanism involving redox potential of these proteins [59] and protein phosphorylation [57].

1.2.5. Extraction of recombinant protein from algae extract

There are many types of cell disruption techniques depending on the type of cell. Most of the cells have certain characteristics which guide the selection of cell disruption technique to be used. For instance, animal cells like CHO cells can be broken easily compared to plant tissues which are more difficult to disrupt due to presence of cellulosic cell wall [60]. Physical disruption of high-water content biomass, such as *Lemna*, plant cell culture, and algae, can be accomplished by using high-shear mixers and/or high pressure homogenizers in the presence of buffer [61]. Various cell disruption techniques tested for algae include homogenizers, ultrasonic, french press [62]. Efficient cell disruption is crucial for maximum recovery of the target protein in the extract. A range of tissue to buffer ratio is used (1:1.5 to 1:10). Lower ratios are preferred as they reduce the process volume, but it should be carefully chosen so as to prevent the loss in the recovery of recombinant protein.

Extraction of recombinant protein is often accompanied by impurities like DNA, chlorophyll and other pigments, host cell proteins, alkaloids, phenolics, polysaccharides,

and proteases. Extraction optimization is done to reduce/remove these impurities from the extract as these components can bind or degrade the recombinant protein affecting final product quality and yield or reduce purification efficiency due to resin or membrane fouling. Also, extraction optimization can protect the recombinant protein from degradation by proteolysis or phenol oxidation [61, 63-68].

Depending on the characteristics of source biomass, target protein and cell disruption technique, different components are present in the extraction buffer. Generally phosphate buffers are used for basic pH extraction of recombinant proteins from green tissue, acetate buffer for acidic pH extraction, and Tris buffer for neutral pH [22, 69]. Detergents like Triton X-100, Tween are added for extraction of recombinant proteins from membrane associated proteins [64] and chloroplast expressed proteins [70, 71]. Protein stabilizers such as: protease inhibitor, antioxidant, and metal chelating agents EDTA, are often added to the extraction buffers to prevent protein degradation. Extraction pH, temperature and extraction time are other factors which affect extraction efficiency and product quality [61].

1.2.6. Primary recovery methods

Development of flexible alternative platform and advancement in upstream processing has led to the shift in focus on downstream processing to alleviate the bottlenecks to process large upstream output. Primary recovery is the first unit operation in a downstream process aimed at removal of cells and cell debris from the culture broth and clarification of the cell culture supernatant that contains the product. The efficiency

of chromatographic separations is largely dependent on initial steps involving precipitation procedures. The clarification step is traditionally performed by centrifugation and filtration. However, the presence of the high solid content and heterogeneous solution of soluble and insoluble components in the fermentation broth makes the primary recovery a significant challenge. Hence there is a dire need to explore additional precipitation/flocculation based pretreatment for improving clarification, yield, and process robustness.

The pretreatments are based on selective precipitation of target protein or host impurities. The number and distribution of charges, hydrophilic and hydrophobic residues on the surface of the protein molecule, size and shape of the molecule are some key features that determine its solubility and hence its tendency to precipitate in a given solvent (Figure 2). The protein solubility can be altered by manipulating the solvent properties like it's ionic strength, pH, and by addition of organic solvents, polymers or combination of these together with temperature variation [72].

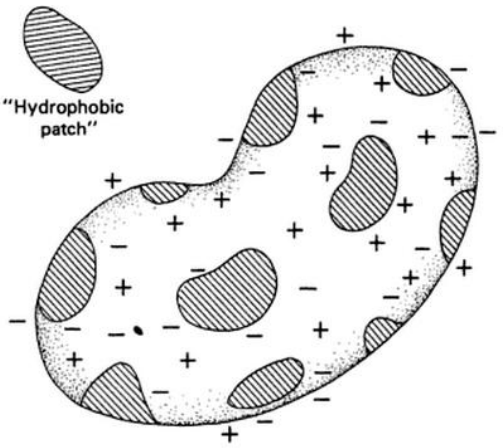

Figure 2. Distribution of charge and hydrophobic patches over surface of a typical protein.

The most common type of precipitation for proteins is salt induced precipitation. At low concentration of the salt, solubility of the proteins usually increases slightly (salting in). But at high concentrations of salt, the solubility of the proteins decreases sharply (salting out) and the proteins precipitate out. The addition of salt in high concentration diminishes electrostatic repulsion between similarly charged groups at the protein surface and disturbs the structure of water molecules around the protein molecule. Salt ions compete with protein globules for water and, eventually, at a sufficiently high concentration, strip the latter of aqueous shell, resulting in protein precipitation. The most effective salts are those with multiple-charged anions such as sulfate, phosphate, citrate; for cations monovalents ions like NH_4^+, Na^+ are used. Because of higher solubility in the wide temperature range of 0-30°C and lower density of the saturated solution (in comparison with the other salts), ammonium sulfate is preferred over other useful salts [73]. Ammonium sulfate precipitation has been used to

condition green tissue extracts including tobacco leaves extract for purification of IgG [74], tobacco cell suspension for purification of GFP-fusion protein [75], rice cell suspension for purification of human serum albumin (HSA) [76], etc. It removes native host cell proteins, rubisco, aggregates, and cell debris [77].

Another method of precipitation is isoelectric precipitation by altering the pH of the solution. At the isoelectric point of the protein, where the net charge on a protein is zero, the electrostatic repulsions between molecules are at a minimum and result in aggregation due to predominating hydrophobic interactions. At a pH below their pI, proteins carry a net positive charge; above their pI they carry a net negative charge. The pI of different proteins is different, which imparts selectivity in precipitation. The various factors which affect the precipitation include the stability of the target protein at the desired pH, the choice of acid or base and the way of addition of acids or bases [73]. Acidic precipitation (isoelectric) has been used for removing phenolics and green pigments from *Lemna* extracts containing IgG [63, 69], reducing phytic acid concentration in rice seed extracts containing human lysozyme [78], reducing rubisco and green pigments of tobacco leaves extracts containing IgG [79].

Flocculation by polymer precipitation has been another popular alternative to salt and pH based precipitation. Proteins can be precipitated with nonionic water soluble polymers like PEG and synthetic or natural polyelectrolytes like PEI or chitosan respectively. There are various mechanisms involved in polymer precipitation but the basic principle is mainly based on electrostatic attraction. Polyelectrolytes absorb to a particle (cell and cell debris) to create an oppositely charged patch on its surface. This

patch can then adhere to a bare patch on an opposing particle surface due to electrostatic attraction [73]. Strongly cationic polymers are more effective at flocculating cells, whereas neutral and anionic polymers are often ineffective [80]. The charge, MW and concentration of the polyelectrolyte, ionic strength, pH and concentration of macromolecule are crucial factors in polymer precipitation [73]. Polyelectrolyte precipitation with polyethyleneimine (PEI) has been successful in removing large amounts of native tobacco impurities as well as ensuring a high recovery and concentration of an acidic recombinant protein from tobacco [81].

Chitosan is another popular flocculant used in extract clarification due to a number of advantages such as it is produced from non-mammalian sources (typically arthropod shells), and is inexpensive and available in a highly purified form that is low in heavy metals, volatile organics and microbial materials. It is a cationic linear polymer of beta-(1–4) linked D-glucosamine monomers generated by the chemical deacetylation of chitin [80, 82, 83]. Chitosan, has been used for the removal of nucleic acids [84], the defatting of protein hydrolysates [85], the flocculation of E. coli cell debris and cell homogenate [83, 86] and the flocculation of yeast [87], bacteria [82] and algae [88]. There is a notable effect of chitosan concentration, sodium chloride concentration and pH on chitosan flocculation of cells, cell debris and particulates [89]. The underlying mechanism of flocculation likely involves electronic interaction between chitosan and charged cellular debris, followed by additional interactive forces such as hydrogen bonding [80].

1.3. Objectives

The objective of the proposed research was to develop technologies and processes for efficient production and purification of single chain antibody fragment from chloroplast of the eukaryotic algae *C. reinhardtii*. This project determined the process conditions and evaluated bioprocessing challenges for algae growth, expression, extraction and purification of single chain antibody from *C. reinhardtii*. The specific objectives of the research study were:

1. To optimize cultivation conditions of *C. reinhardtii* to achieve maximum growth rate and accumulation of αCD22 scFv and Pfs25 in its chloroplast.
2. To optimize light induction to achieve maximum accumulation of αCD22 scFv in the chloroplast.
3. To evaluate extraction conditions for maximum recovery of αCD22 scFv in the algae extract.
4. To evaluate different primary recovery methods for clarification of algae extract.

CHAPTER II

LIGHT-INDUCED PRODUCTION OF AN ANTIBODY FRAGMENT AND MALARIA VACCINE ANTIGEN FROM *CHLAMYDOMONAS REINHARDTII**

2.1. Introduction

The possibility of producing complex and diverse therapeutic proteins, such as monoclonal antibodies, antibody conjugates and vaccine antigens, in the chloroplast of *Chlamydomonas reinhardtii* has been reported [3-6, 16, 90]. The potential of microalgae to produce recombinant protein was previously reported [4-6, 16, 90]. Simple growth requirements, rapid growth and scalable production makes the unicellular eukaryotic green algal, *Chlamydomonas reinhardtii*, an attractive alternative, especially for complex therapeutic proteins that are not efficiently expressed in other heterologous systems. The expression of molecules that are not glycosylated or that do not require glycosylation for function are best suited for chloroplast expression in *C. reinhardtii*. Examples include antibody fragments, anthrax toxin blocking IgG [70], immunotoxins and transmission-blocking malaria vaccines (TBV) [91]. Although the expression, authenticity and activity of complex recombinant proteins in microalgae chloroplast are important prerequisites, one has to establish that these molecules can be produced at a

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competitive cost [61]. The aim of this study was to identify and evaluate variables that affect the accumulation and extraction of recombinant proteins produced in algal chloroplast.

Plasmodium falciparum surface protein 25 (Pfs25TBV/Pfs25) [5] and single-chain antibody fragment (αCD22 scFv) [6] were chosen as representative therapeutic protein molecules that have been produced in the chloroplast of *C. reinhardtii*. Pfs25 is a structurally complex, aglycosylated outer membrane protein and a leading subunit TBV candidate for malaria. The chloroplast of *C. reinhardtii* is a particularly attractive location for the production of aglycosylated therapeutic molecules, like Pfs25, that require eukaryotic-like machinery for proper folding and disulfide bond formation. The antibody fragment (αCD22 scFv) recognizes the CD22 B-cell surface epitope and has been used to generate fully-functional antibody-toxin chimeric proteins [6, 21]. Expression of these genes is regulated by the psbA promoter in psbA-deficient *C. reinhardtii* strains, which are non-photosynthetic, because the psbA gene product D1 is required for photosynthesis. This specific promoter and strain combination was made because it resulted in the highest levels of recombinant protein accumulation in *C. reinhardtii* [19, 23] and allows for the decoupling of heterotrophic growth and light-induced gene expression.

While many studies in *C. reinhardtii* have demonstrated light-induced gene expression [92-94], few have demonstrated the profound effect it can have on recombinant protein accumulation. Previous studies [95] established that a high cell density reduces light transmission in growing cultures and negatively affected

recombinant protein accumulation. Recent reports indicate that, in addition to the cell shading effect (reduced light transmission), the size of the synthesized molecule is also an important light induction variable. For example, a 5–8-h light induction time was used for Pfs25 using a photon irradiance of 5000 lux (68 μmol m^{-2} s^{-1} [5], whereas the CtBx-Pfs25 fusion protein required a 24-h induction time at the same irradiance level [96]. The larger of the two proteins, a monoclonal antibody-toxin conjugate, required a 96-h light exposure at 10,000 lux (135 μmol m^{-2} s^{-1}) [6]. Therefore, the effect of light exposure conditions (time and irradiance levels) on recombinant protein accumulation is a critical issue.

The objective of this study is to provide a quantifiable account of light-induced recombinant protein accumulation in transgenic *C. reinhardtii*. Specifically, we wanted to determine the process conditions that could maximize *C. reinhardtii* biomass concentration and recombinant protein accumulation. Pfs25 (25 kDa) and αCD22 scFv (30 kDa) have similar molecular weights and, thus, reduce the potential effect of molecular size on light-induced accumulation.

2.2. Materials and Methods

2.2.1. Gene constructs for αCD22 scFv and Pfs25

In both constructs, the endogenous psbA locus was replaced by Pfs25 or αCD22 scFv via direct homologous recombination. Thus, transgene expression in these strains is regulated by the psbA promoter and the 5' and 3' untranslated regions (UTRs) and,

therefore, is light inducible. A kanamycin resistance cassette was incorporated for selection. In the case of αCD22 scFv, the variable domains of a human antibody against the B-cell surface antigen CD22 were separated by a linker consisting of four glycines and a serine repeated four times (4× G4S) to create an scFv [6]. Both of the gene cassettes (αCD22 scFv and Pfs25) were ligated with a sequence coding for a 1× FLAG peptide (DYKDDDDKS) and separated by a sequence that encodes a tobacco etch virus (TEV) protease cleavage site (ENLYFQG) [5].

2.2.2. Cultivation of recombinant Pfs25 and αCD22 scFv *Chlamydomonas reinhardtii* strains

Algal biomass from a single agar plate (Tris-Acetate-Phosphate TAP agar with 150 μg/mL kanamycin) was transferred to 100 mL of TAP media without kanamycin and grown for 3 days. A subsequent volumetric culture scale up was performed using 10% inoculum in the exponential phase (100 mL) in 1 L of fresh TAP media containing 25 μg/mL kanamycin. One-liter cultures were grown heterotrophically (in the dark) for 5 days, reaching ~4 to 5 × 10^5 cells/mL. For resuspension experiments at the end of the fifth day, the biomass from 1-L cultures was resuspended in 1-L of fresh TAP media containing 25 μg/mL kanamycin and grown for 1 day, reaching about 10^6 cells/mL. The latter cultures were then exposed to light to induce recombinant protein synthesis. Control experiments without resuspension were performed under the same time and light regimes. For each recombinant protein, three replicate batches were grown in different conditions (resuspension vs. non-resuspension) followed by light induction. Cell growth

and cell concentration were monitored daily by counting cells using a hemocytometer (Bright Line, Hausser Scientific, Horsham, PA, USA) and by measuring the optical density at a 750-nm wavelength using a DU640 spectrophotometer (Beckman Coulter, Brea, CA, USA).

2.2.3. Effect of light duration and light intensity on light-induced production of αCD22 scFv

The effect of photon irradiance flux on the accumulation of αCD22 scFv was evaluated in cultures grown under heterotrophic conditions followed by resuspension to a final cell concentration of ~10^6 cells/mL, as described above. Cultures were exposed to light for 12 h and 24 h consecutively at a photon irradiance of 101 μmol m^{-2} s^{-1} and 300 μmol m^{-2} s^{-1}. Recombinant protein was extracted and quantified by anti-FLAG affinity purification, as described below.

2.2.4. Protein extraction

C. reinhardtii cultures producing recombinant proteins were grown in liquid media until they reached the desired cell concentration of ~10^6 cells/mL. At the end of the light exposure period, cells were harvested by centrifugation at 10,000× g for 15 min at 4°C. Pelleted algal biomass was washed with fresh TAP media, weighed and then resuspended at a 1:5 biomass-to-lysis buffer ratio (50 mM Tris-HCl, 400 mM NaCl and 0.5% Tween, pH 8.0). The buffer contained a complete protease inhibitor cocktail (Roche, Mannheim, Germany) dissolved in 200 mL of the buffer. Algal cells were lysed

by sonication for 8 min with 30 s on/off intervals at 4 °C using a sonicator (Sonifier 250, Branson, Danbury, CT, USA) at 30% output control and 30% duty cycle with a micro probe (1/8" microtip A3-561 Branson, Danbury, CT, USA). Cell lysates were centrifuged (10,000× g for 10 min) to produce clarified crude extracts.

2.2.5. Protein analysis

Filtered algal crude extract and purified samples were analyzed by SDS-PAGE and western blot, and the total eluted protein was determined by the Bradford assay [97]. Total soluble protein from crude extracts and purified samples were quantified using the microplate protocol (working range from 1 to 25 μg/mL and 25 to 1,500 μg/mL) Coomassie plus (Bradford) assay kit (Thermo Scientific, Waltham, MA, USA). Absorption at 595 nm was measured using the VERSA max microplate reader (Molecular Devices, Sunnyvale, CA, USA).

NuPAGE Novex Bis-Tris pre-cast gradient gels (4%–12%) from Invitrogen™ (Carlsbad, CA, USA) (1.5 mm × 10 wells), (Cat No. NP0335BOX) were used for SDS-PAGE electrophoresis. Reducing buffer was prepared using LDS sample buffer (4×) (NuPAGE NP0007) containing 10% of reducing agent (Cat No. NP0004). Reduced samples were prepared using a 1:4 ratio reducing-buffer: sample and heated at 70°C for 10 min. MES SDS Running Buffer (20×) (Cat No. NP0002) stock solution was used to prepare 1× running buffer in reverse osmosis water. Antioxidant (NuPAGE NP0005, Invitrogen™, Carlsbad, CA, USA) was added to ensure reduced samples during electrophoresis. Gels were run for 35 min at a constant voltage (200 V). For SDS

analysis, the gels were stained in Coomassie™ (Thermo Fisher Scientific, Waltham, MA, USA) G-250 stain (Cat No. LC6065) for 3 h, followed by destaining in RO water. For western blot analysis, the gel was transferred to nitrocellulose membranes using the iBlot® 7-Minute Blotting System, Life Technologies Corporation (Carlsbad, CA, USA).

After protein transfer to a nitrocellulose membrane, the membrane (free sites) was blocked with 2.5% non-fat milk in TBS containing 0.05% Tween 20 at pH 7.5 buffer for 1 h to prevent nonspecific binding of the detection antibodies. FLAG-tagged recombinant proteins (αCD22 scFv and Pfs25) were detected by using anti-FLAG M2-AP (alkaline phosphatase conjugated) antibody from Sigma Aldrich (Cat No. A9469, St. Louis, MO, USA) at a concentration of 1:1000. After incubation with the antibody for 1 h, the membrane was washed with TBS containing 0.05% Tween 20 at pH 7.5; buffer and blots were visualized (developed) with nitro-blue tetrazolium (NBT) and 5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt (BCIP) (Sigma FAST B5655, St. Louis, MO, USA) dissolved in 10 mL of filtered RO water.

2.2.6. FLAG affinity purification

Crude extracts were filtered using a polyethersulfone (PES) 0.45-μm filter and mixed with anti-FLAG affinity resin (Sigma Aldrich A4596, St. Louis, MO, USA) equilibrated in the same lysis buffer used for protein extraction. Approximately 1 mL of resin was used per every 4 g of wet algal biomass. Binding of the recombinant protein to the affinity resin was performed for 2 h at 4°C by continuous end-over-end mixing in a Glas-Col rotor (Glas-Col LLC, Terre Haute, IN, USA) at ~33 rpm (40% speed control).

Affinity resin was washed with 10 column volumes (CV) of lysis buffer followed by 3 column volumes of lysis buffer without Tween. The washed FLAG resin was transferred into Bio Spin disposable chromatography columns (Bio Rad, Cat No. 732-6008, Hercules, CA, USA) for protein elution at room temperature. Recombinant protein was eluted at pH 3.5 using 5 CV of 100 mM glycine buffer, pH 3.5 that contained 400 mM NaCl. Eluted protein fractions were collected in 5 tubes containing a predetermined amount of 1M Tris-HCl, pH 8.0, to immediately increase the pH of the eluted protein and avoid protein denaturation. Typically, three elution fractions (E2 to E4) were used for the estimation of purity and yield, although some losses occurred by not taking into account E1 (Elution Fraction 1). By pooling these three fractions, more than 80% of extracted FLAG-tagged proteins were recovered. Extraction buffer and all of the materials used, including the sonication probe (1/8" microtip A3-561 Branson, Danbury, CT, USA), were cooled in advance.

The FLAG affinity purification method was used as a convenient analytical tool to determine the recombinant extraction yield. The resin was added in sufficient amounts to bind all available FLAG fusion protein present in clarified extracts. Cell debris and supernatants at the end of the batch adsorption period were regularly analyzed by western blotting to assure complete extraction and adsorption, respectively. Although minor recombinant protein losses have occurred during resin washing and pH 3.5 elution from the anti-FLAG resin, this determination of recombinant protein concentration was considered appropriate for estimating recombinant protein in crude extracts.

2.2.7. Statistical analysis

Design Expert software (Version 9, Stat-Ease, Inc., Minneapolis, MN, USA, 2014) was used for the experimental design and analysis. The statistical significance of the models was evaluated by the analysis of variance (ANOVA). Effects with more than 95% of significance (95% confidence interval), that is effects with a *p*-value lower than 0.05, were significant. Significantly different means ($p < 0.05$) were separated by Tukey's test.

2.3. Results and Discussion

2.3.1. Algae cultivation and accumulation of αCD22 scFv and Pfs25

The optimization of algal growth and induced expression conditions could significantly enhance recombinant protein accumulation. Maximal biomass accumulation was achieved after four to five days of continuous growth under heterotrophic conditions (no light exposure) with the cell concentration reaching 5 to 6 × 10^5 cells/mL (Figure 3). In order to further increase the cell concentration and to provide sufficient nutrients for the subsequent induction phase, cells were resuspended in fresh media at the end of the fifth day and allowed to grow for 24 h before the induction (light exposure) period (Figure 3).

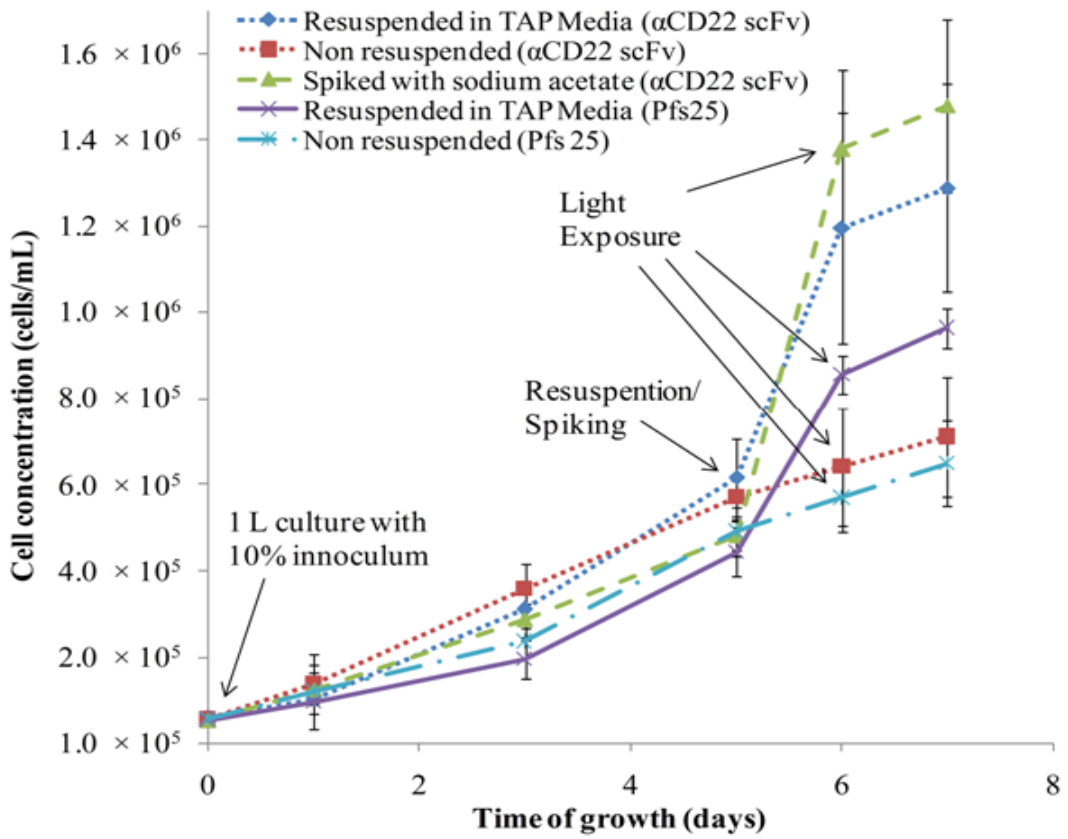

Figure 3. Heterotrophic growth curves of *Chlamydomonas reinhardtii* expressing αCD22 scFv and Pfs25. The effect of resuspension, spiking and light exposure (101 µmol $m^{-2}s^{-1}$) on cell concentration. Values indicated are average of 3 replicates. TAP: Tris-Acetate-Phosphate.

Twenty-four hours after resuspension (Day 6), the cell number of resuspended cultures increased almost two-fold (1.8-fold for αCD22 scFv and 1.5-fold for Pfs25) compared to non-resuspended ones (Figure 3). The exposure of the cultures to light for 24 h (Day 7) increased cell concentrations of resuspended and non-resuspended cultures proportionally, maintaining the two-fold difference. The variability in cell concentrations for resuspended cultures could be due to different cell adaptation periods between (lag phase) the three batches after resuspension.

We tested both 24 h and 48 h incubation after resuspension and found that 24 h was optimal, as no additional increase in cell number was observed. The increase in culture pH to 8.0 near the end of the exponential phase (Day 5) reflects the depletion of acetate (carbon source); therefore, the increase in cell number during resuspension could be attributed to the replenishment of the carbon source in the media. We confirmed the above hypothesis by spiking the culture with sodium acetate (1.68 g/L), which also resulted in about a two-fold increase in the cell concentration. The growth curve of *C. reinhardtii* after spiking with sodium acetate followed a similar trend as resuspension with fresh TAP media (Figure 3). Thus, cell resuspension is not necessary, and fed-batch cultivation with only acetate addition would be a feasible method to increase algal biomass. At the end of Day 6, wet biomass of resuspended cultures increased 2.65-fold for αCD22 scFv and three-fold for Pfs25 compared to the non-resuspended cultures. The reason for the higher fold increase in wet biomass compared to cell concentration reflects the increase in the cell mass (i.e., cell size) with resuspension.

Total biomass, as measured by optical density at 750 nm, as well as extracted soluble protein (TSP) in cultures increased with increased light exposure, which agrees with the fact that RuBisCO and other light-activated enzymes and pigments are being synthesized under light conditions [50]. In *C. reinhardtii*, the genes encoding the light harvesting chlorophyll a/b-binding proteins (LHCPs) and the oxygen evolving enhancer (OEE) complex proteins are expressed when cells are shifted from the dark into white light [98]. During light exposure, there was no significant increase in cell concentration,

probably due to a higher energy demand for carbon fixation, since the carbon uptake flux is directed through the Calvin cycle [50, 99].

We investigated the effect of resuspension and light induction on αCD22 scFv and Pfs25 synthesis and accumulation, because the aim of increasing the cell density was to increase the recombinant protein accumulation per liter of culture. The effect of resuspension on the amount of purified αCD22 scFv and Pfs25 is shown in Figure 4a. The increase of cell concentration resulted in a 2.8-fold increase of αCD22 scFv and a 2.8-fold increase of Pfs25 protein eluted from the affinity resin.

These results also suggest a correlation between total soluble protein and recombinant protein accumulation in crude extracts (Figure 4b). Because resuspension has a positive effect on the overall growth of the algal cells, replenishment of the media also results in an increase in total soluble protein. In the case of αCD22 scFv, the increase in purified recombinant protein amount was synchronous with the increase in total soluble protein (TSP). As expected, resuspension resulted in a similar fold increase in recombinant protein accumulation (2.8 ± 0.9) and total soluble protein accumulation (2.9 ± 0.4). However, Pfs25 showed a greater fold increase in purified recombinant protein concentration (2.8 ± 0.5) compared to the (1.1 ± 0.2) fold increase in total soluble protein accumulation. The higher fold increase in the FLAG-purified Pfs25 protein concentration compared to αCD22 scFv might have been the result of Pfs25 aggregation (Figure 5a, b), which often reduces recombinant protein susceptibility to proteases [100].

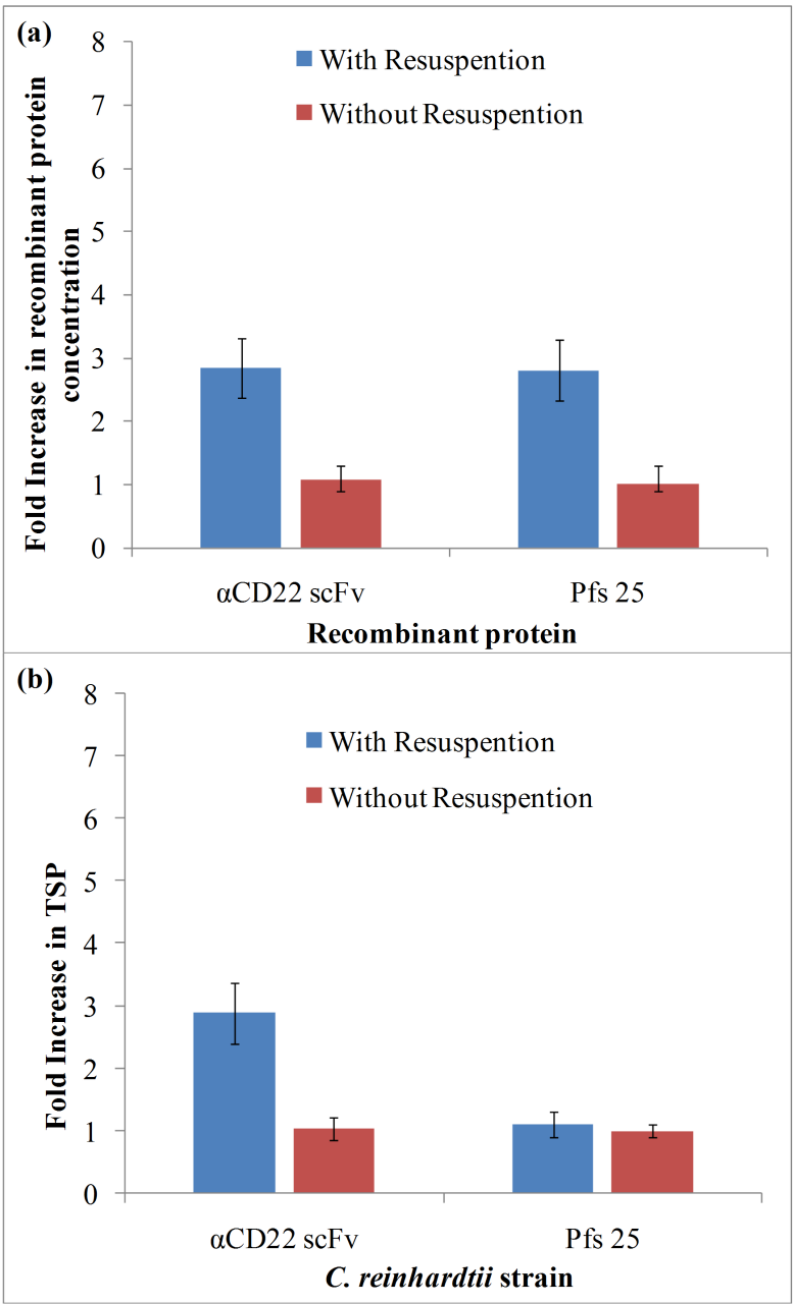

Figure 4. The effect of resuspension on (a) purified recombinant protein (αCD22 scFv and Pfs25) and (b) total soluble protein concentrations. Values indicated are average of 3 replicates.

2.3.2. Purification and analysis of recombinant proteins

Recombinant proteins were purified from clarified crude lysates by FLAG affinity adsorption and analyzed by SDS-PAGE and western blotting (Figure 5a, b). The presence bands of negligible intensity in the supernatant after 2 h of incubation (Lane 3) and washes before elution (Lanes 4–5) at the same size as Pfs25 confirm the efficient binding of FLAG-tagged Pfs25 to the anti-FLAG affinity resin. SDS-PAGE gels and total eluted protein analysis by the Bradford assay indicated that the majority of purified Pfs25 (25 kDa band) eluted in Fractions 2, 3 and 4 (Lanes 7–9). These two fractions typically contained approximately 80% of the total eluted recombinant protein from the resin. The eluted fractions, in both the SDS-PAGE and western blot, show the presence of aggregates, even under reducing conditions (~50 kDa to 100 kDa bands) in Figure 5a.

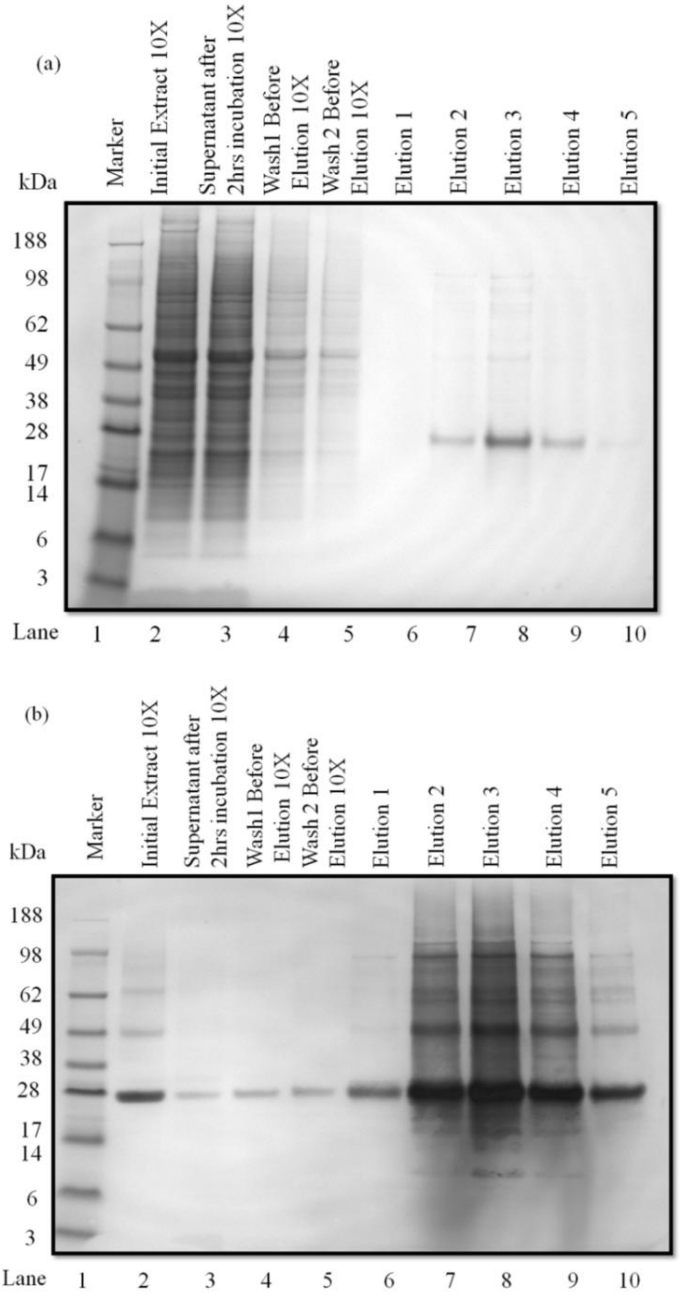

Figure 5. (a) Coomassie-stained SDS-PAGE of reduced *C. reinhardtii* Pfs25 purified with FLAG affinity chromatography. Lane 1: molecular weight marker (kDa); Lane 2: 10X diluted clarified algae extract; Lane 3: 10X diluted supernatant after 2 h of binding with FLAG resin; Lanes 4–5: washes before elution; Lanes 6 to 10: pH 3.5 eluted fractions; (b) Western blot analysis of *C. reinhardtii* Pfs25 using anti-FLAG-AP conjugated antibody. Lane 1: molecular weight marker (kDa), Lane 2: 10X diluted clarified initial extract; Lane 3: 10X diluted supernatant after 2 h of binding with FLAG resin; Lanes 4 and 5: washes before elution, Lanes 6 to 10: pH 3.5 eluted fractions.

The detection of aggregates was not surprising, because Pfs25 is a membrane protein, and similar aggregation has also been observed in yeast-produced Pfs25 [101, 102]. In spite of Pfs25 aggregation, FLAG-affinity purified Pfs25 multimers generated an immune response and elicited antibodies with significant levels of transmission blocking activity [5].

The analyses of purified αCD22 scFv revealed no significant aggregation in the eluted fractions by SDS-PAGE (Figure 6a, Lanes 6 to 10) and western blot (Figure 6b). Similar to Pfs25, the insignificant losses in the supernatant (Lane 2) and washes (Lanes 3–4) confirms the efficient binding and elution of FLAG-tagged αCD22 scFv from the anti-FLAG affinity resin. A major 30-kDa band was detected by the SDS-PAGE gel (Figure 6a) and western blot (Figure 6b). Western blot (Figure 6b, Elution 2) revealed the presence of minor degradation products of 14 kDa and 17 kDa in size.

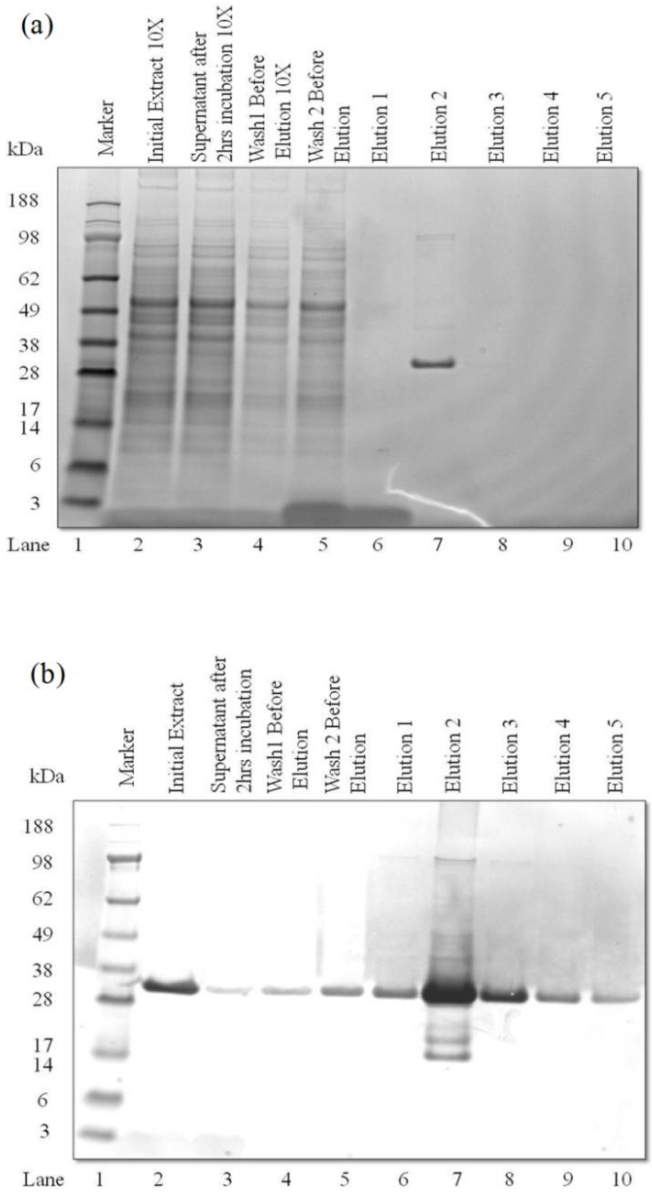

Figure 6. (a) Coomassie-stained SDS-PAGE gels of reduced *Chlamydomonas reinhardtii* αCD22 scFv purified with FLAG affinity chromatography. Lane 1: molecular weight marker (kDa); Lane 2: 10× diluted clarified initial extract; Lane 3: 10× diluted supernatant after 2 h of binding with FLAG resin; Lane 4: wash 1 before elution 10×; Lane 5: wash 2 before elution; Lanes 6 to 10: pH 3.5 eluted fractions; (b) Western blot analysis of C. *reinhardtii* CD22 scFv using anti-FLAG-AP conjugated antibody. Lane 1: molecular weight marker (kDa); Lane 2: clarified initial extract; Lane 3: supernatant after 2 h of binding with FLAG resin; Lane 4: wash 1 before elution; Lane 5: wash 2 before elution; Lanes 6 to 10: pH 3.5 eluted fractions.

2.3.3. Light-induced accumulation of αCD22 scFv

We observed that the recombinant protein accumulated did not increase significantly when the wet biomass concentration increased beyond 4 g/L (>10^6 cells/mL), and some batches even reported somewhat lower recombinant protein accumulation. The significant ($p < 0.05$) increase in the cell number that occurred after resuspension raised the question of the cell shading effect on protein accumulation. Because light induction is a unique feature of psbA-driven gene constructs, we hypothesized that recombinant production would be affected by the amount of light (total energy) reaching each cell in the culture. To determine the optimal light conditions required to overcome shading and maximize recombinant protein accumulation, we used only αCD22 scFv strain, because extensive aggregation of Pfs25 could obscure the effect of delivered light flux on the recombinant protein yield.

A 2^3 full factorial design was performed to determine the effect of the light intensity, light duration and light placement on the volumetric concentration of recombinant protein αCD22 scFv. The optimal cell concentration used for light induction was 1×10^5 cells/mL. The placement of the light source (one vs. two sides) did not have a significant effect on the recombinant protein accumulation; however, there was a significant effect ($p < 0.05$) of light duration and light intensity on recombinant protein accumulation. Because preliminary data with both Pfs25 and αCD22 scFv showed no substantial protein accumulation beyond 24 h of light exposure at 101 μmol m^{-2} s^{-1}, we restricted the further investigation to 12 h and 24 h of light exposure and two levels of light intensity: 101 μmol m^{-2} s^{-1} and 300 μmol m^{-2} s^{-1}. The effect of these two

variables is summarized in Table 1. It is evident from the data that a 24-h light duration at both light intensities was optimal for maximum recombinant protein accumulation per gram of wet biomass and unit culture volume. At a light intensity of 101 μmol m^{-2} s^{-1}, 21.2 ± 1.8 μg/g of αCD22 scFv was recovered after 12 h of light exposure compared to 35.1 ± 5.8 μg/g after 24 h of light exposure. A similar time effect was observed at 300 μmol m^{-2} s^{-1}; 26.6 ± 4.3 g/g of αCD22 scFv were recovered after 12 h and 61.5 ± 14 μg/g after 24 h. Interestingly, the recombinant protein recovered after 12 h of exposure at a higher light intensity of 300 μmol m^{-2} s^{-1} (26.6 ± 4.3 μg/g) was not significantly different from the 12 h of light exposure at 101 μmol m^{-2} s^{-1} (21. 2 ± 1.8 μg/g). However, after 24 h, the difference in αCD22 scFv accumulation at 300 μmol m^{-2} s^{-1} became twice as much as that at 101 μmol m^{-2} s^{-1}, indicating that both the light intensity and duration of exposure were important factors for the synthesis of recombinant protein. The same conclusion could be reached by comparing αCD22 scFv volumetric concentrations after 12 and 24 h at 300 μmol m^{-2} s^{-1} and 101 μmol m^{-2} s^{-1}, respectively. Interestingly, the effect of the same two factors was different for total soluble protein. Host proteins, such as RuBisCO and other light-activated enzymes listed under TSP in Table 1, were synthesized mainly during the first 12 h of light exposure, and the TSP concentration did not significantly change during the next 12 h. These data suggest that native host proteins were synthesized faster than the recombinant protein and that the 12-h period of light exposure was not sufficient for maximal accumulation of αCD22 scFv. These results corroborate previous data indicating that the translation of the psbA promoter is a rate-limiting step for recombinant protein expression in transgenic *C.*

reinhardtii [52]. The increase of αCD22 scFv accumulation (% TSP) after 24 h at both light intensities (Table 1) reflects the effect of light duration and intensity on continued αCD22 scFv accumulation at constant total soluble protein. The two-fold increase of the recombinant protein fraction in the extracted soluble protein is a desirable product recovery outcome, which is equivalent to achieving a two-fold purification [61].

Table 1. The effect of light intensity and duration on biomass harvested, total soluble protein (TSP) and αCD22 scFv protein production. The values given are averages from three replicates ± standard deviations. a,b For each observation, means within a column that are not followed by a common superscript letter are significantly different ($p < 0.05$); x,y means within a row that are not followed by a common subscript letter are significantly different ($p < 0.05$).

Photosynthetic Photon Flux (PPF)	**Duration**	**101 µmol m^{-2} s^{-1}**	**300 µmol m^{-2} s^{-1}**
Biomass (g)	12 h	$_{x}$ 4.0 ^{a} ± 0.01	$_{x}$ 4.9 ^{a} ± 1.5
	24 h	$_{x}$ 4.0 ^{a} ± 0.01	$_{x}$ 5.3 ^{a} ± 1.0
TSP in algae extract (µg/mL)	12 h	$_{x}$ 5939 ^{a} ± 257	$_{x}$ 5485 ^{a} ± 802
	24 h	$_{x}$ 6840 ^{a} ± 1167	$_{x}$ 5248 ^{a} ± 415
αCD22 scFv in wet biomass (µg/g)	12 h	$_{x}$ 21.2 ^{a} ± 1.8	$_{x}$ 26.6 ^{a} ± 4.3
	24 h	$_{x}$ 35.1 ^{b} ± 5.8	$_{y}$ 61.5 ^{b} ± 14
αCD22 scFv volumetric conc. (µg/L)	12 h	$_{x}$ 84.7 ^{a} ± 7.1	$_{x}$ 135 ^{a} ± 66
	24 h	$_{x}$ 140 ^{a} ± 20	$_{y}$ 314 ^{b} ± 20
αCD22 scFv (%TSP)	12 h	$_{x}$ 0.07 ^{a} ± 0.01	$_{x}$ 0.1 ^{a} ± 0.03
	24 h	$_{x}$ 0.12 ^{a} ± 0.03	$_{y}$ 0.23 ^{b} ± 0.05

2.4. Summary

This study provides an account of factors that affect the accumulation of two recombinant proteins in *C. reinhardtii* chloroplast. Cultivation variables of *C.*

reinhardtii, such as light-induction time and intensity, replenishment with fresh media and culture age, had a significant effect on biomass and recombinant protein accumulation.

Maximum biomass accumulation of transgenic *C. reinhardtii* was achieved after resuspending the cells in fresh TAP media after five days of heterotrophic growth, which resulted in a 2.8-fold increase in the culture concentration of recombinant therapeutic proteins αCD22 scFv and Pfs25, but this could also be achieved by adding acetate. There was a correlation between total soluble protein and recombinant protein accumulation in crude extracts for αCD22 scFv, but not Pfs25. Resuspension resulted in a similar fold increase in αCD22 scFv accumulation (2.8 ± 0.9) and total soluble protein accumulation (2.9 ± 0.4).

There was also a significant effect of light duration and intensity on the psbA regulated expression of αCD22 scFv protein in the chloroplast. The exposure of light of 300 μmol m^{-2} s^{-1} for 24 h resulted in a maximum culture concentration of αCD22 scFv (314.0 ± 20 μg/mL), which is a two-fold increase compared to 101 μmol m^{-2} s^{-1}. Decoupling of the cell growth and light-induced production of Pfs25 and αCD22 scFv allowed us to demonstrate that native *C. reinhardtii* proteins accumulate faster (12 h) during the light phase than recombinant proteins, which needed 24 h of light exposure. Thus, an increase in light intensity to 300 μmol m^{-2} s^{-1} not only increased the recombinant protein production, but also led to an efficient product recovery with a two-fold increase of the recombinant protein fraction in the extracted soluble protein.

The two proteins were efficiently purified by FLAG affinity adsorption with minimal losses (less than 20%) as judged by western blots. Overall, resuspension and light optimization resulted in a six-fold increase in the recovered recombinant protein in the chloroplasts of *C. reinhardtii*.

CHAPTER III

EVALUATION OF EXTRACTION CONDITIONS AND PRETREATMENT METHODS FOR PRODUCTION OF AN ANTIBODY FRAGMENT FROM *CHLAMYDOMONAS REINHARDTII*

3.1. Overview

Advancement in upstream processing titers and introduction of novel platforms for recombinant protein production has led to more complex product feed streams to chromatography (culture broths and cell lysates). Because downstream processing of therapeutic protein products can account for a significant portion of the total manufacturing costs, the development of efficient and selective recovery and purification processes as early as possible is essential for favorable economics [94].

Primary recovery is the first stage in downstream processing aimed at clarification of cell culture and cell lysates to yield clarified feed suitable for chromatography. Traditionally, downstream processing of therapeutic proteins has been designed to place nearly all of the purification capabilities on chromatography steps with the clarification steps signed exclusively for cell and cell debris removal. Primary recovery steps for therapeutic products typically consist of cell or cell debris removal by centrifugation followed by depth filtration and sterile filtration before capture chromatography. Clarification of cell lysates during recovery of intracellular products could be challenging and the removal cell debris by centrifugation and depth filtration is

often not efficient and, in some cases, inadequate. Recently, primary recovery of monoclonal antibodies from high-titer mammalian cell cultures have faced similar efficiency challenges that led to investigating pretreatment methods (flocculation and precipitation) for improving clarification, yield, and process robustness [80, 103].

Green microalgae are being currently evaluated as a potential platform for expressing variety of non-glycosylated protein products [2, 3, 5, 6, 16, 20, 70] and recombinant protein recovery and purification data are not available. Most recombinant proteins are best expressed and localized in the algal chloroplast and in the absence of other relevant data, one can only assume that optimal target protein extraction and recovery from algal biomass would be similar in many respects to that from transgenic green tissues. The protein release and extraction from transgenic biomass tissue is a critical recovery step because it dictates the total extract volume, recombinant protein concentration and purity, and the type and quantity of impurities that have to be removed during purification [64, 104]. During extraction, recombinant protein is often accompanied by the release of a variety of host cell proteins, DNA and other plant-specific impurities such as chlorophyll pigments, alkaloids, and phenolics. Extraction conditions (temperature, pH, salt, detergents, biomass-to-buffer ratio) have been used to reduce interactions between the recombinant protein and cell debris, to protect recombinant protein from degradation, and hence maximize the recombinant protein yield [61, 64, 66-68, 105]. With exception of phenolics and alkaloids, green microalgae extracts resembles green leaf extracts and generated knowledge could be applicable to transgenic microalgae.

Several pretreatment strategies like aqueous two-phase partitioning [106, 107], ammonium sulfate precipitation [74], isoelectric precipitation [69, 105] and polymer precipitation [84, 108] have been successful with the green tissue extracts. Low percentage ammonium sulfate (25–30%) precipitation is another common method for pretreatment of cell culture and green tissue homogenates [74]. Ammonium sulfate precipitation removed native plant proteins including the photosynthetic protein, ribulose bis-phosphate carboxylase/oxygenase (rubisco), aggregates, and cell debris [77]. In case of acidic precipitation, adjusting the pHs of leafy extracts and cell homogenates to <5.0 precipitates the most abundant plant protein (rubisco), cell debris, as well as chlorophyll pigments attached to the protein and debris [77, 105]. Similar to ammonium sulfate precipitation, acid precipitation resulted in at least two-fold purification of a recombinant protein prior to the first chromatography column. Our lab has previously demonstrated that acidic (pH 4.5) precipitation of *Lemna minor* extracts containing IgG also removed phenolics and green pigments [69, 105].

Cationic polymers, PEI and chitosan, has been successfully used for flocculation of debris, cells and particulates [83, 88, 108]. Chitosan, has several unique properties that make this polysaccharide polymer particularly popular: it is inexpensive (~$60/kg), biodegradable, non-toxic, tolerant to presence of salts in solution, and available as a food grade material that is low in heavy metals and volatile organics. [89]. Chitosan has been used for the removal of nucleic acids [84], the defatting of protein hydrolysates [85], the flocculation of *E. coli* cell debris and cell homogenate [83, 86] and the flocculation of yeast [87], bacteria [82] and algae [109, 110].

Although the pretreatments listed above have been used to reduce DNA, host protein or cell debris, flocculate cells and cell debris, none of the studies quantified the effect of selected pretreatments on debris removal, reduction of DNA, host cell proteins, pigments, etc. In this study we compare three pretreatment methods and quantify their effect on recovery of *s*ingle-chain antibody fragment (αCD22 scFv) [6] produced in the chloroplast of *C. reinhardtii*. Specifically, the aim was to select extraction conditions and pretreatment of lysates and clarified extracts for maximum reduction of impurities load without comprising the recovery of recombinant αCD22 scFv.

3.2. Materials and Methods

3.2.1. Gene constructs for αCD22 scFv

In the construct, the endogenous *psbA* locus was replaced by αCD22 scFv via direct homologous recombination. Thus, transgene expression in these strains is regulated by the psbA promoter and the 5' and 3' untranslated regions (UTRs) and, therefore, is light inducible. A kanamycin resistance cassette was incorporated for selection. The variable domains of a human antibody against the B-cell surface antigen CD22 were separated by a linker consisting of four glycines and a serine repeated four times (4×G4S) to create an scFv [6]. The gene cassettes (αCD22 scFv) was ligated with a sequence coding for a 1× Flag peptide (DYKDDDDKS) and separated by a sequence that encodes a Tobacco etch virus (TEV) protease cleavage site (ENLYFQG) [6].

3.2.2. Cultivation of recombinant αCD22 scFv *C. reinhardtii* strains

Algal biomass from a single Tris acetate phosphate agar plate containing 150 μg/ml kanamycin) was transferred to 100 mL of TAP (Tris acetate phosphate) media without kanamycin and grown for 3 days. Subsequent volumetric culture scale up was performed using 10 % inoculum in the exponential phase (100 mL) in 1-L of fresh TAP media containing 25μg/mL kanamycin. One liter cultures were grown heterotrophically (in the dark) for 5 days reaching ~ 4 to 5 × 10^5 cells/mL. At the end of the fifth day, the biomass from 1-L cultures was resuspended in 1-L of fresh TAP media containing 25 μg/mL kanamycin and grown for 1 day reaching about 10^6 cells/mL. The cultures were then exposed to light at 300 μmol $m^{-2}s^{-1}$ for 24 hours to induce recombinant protein synthesis. Cell growth was monitored daily by counting cells using a hemocytometer (Bright Line, Hausser Scientific, Horsham, PA) and measuring optical density at 750 nm wavelength using a Beckman Coulter (Brea, CA) DU640 spectrophotometer.

3.2.3. Protein extraction

C. reinhardtii cultures producing recombinant proteins were grown in liquid media until they reached the desired cell concentration of ~10^6cells/mL. At the end of the light exposure period, cells were harvested by centrifugation at 10,000 ×g for 15 min at 4 ºC. Pelleted algal biomass was washed with fresh TAP media, weighted, and then resuspended at 1:5 biomass-to-lysis buffer ratio 50 mM Tris-HCl, 400 mM NaCl, and 0.5% Tween 20, pH 8.0. The buffer contained a complete protease inhibitor cocktail (Roche-Mannheim, Germany) dissolved in 200 mL of the buffer. Algal cells were lysed

by sonication for 8 min with 30 s on/off intervals at 4^{o}C using sonicator (Sonifier 250, Branson, USA) at 30% output control and 30% duty cycle with a micro probe (1/8' microtip A3-561 Branson, USA). Cell lysates were centrifuged (10,000 x g for 10 min) to produce cell-free extracts.

For optimization of extraction conditions of αCD22 scFv, buffer composition (pH, NaCl, Tween 20) was varied depended on the extraction objective. Two levels each for pH (4.5 and 8), NaCl (100 mM, 400 mM) and Tween 20 (0% and 0.5%) were tested. All buffers contained a complete protease inhibitor cocktail (Roche-Mannheim, Germany) dissolved in 200 mL of the buffer. Cell lysates were centrifuged (10,000 x g for 10 min) and filtered with PES 0.45 μm syringe filter to produce clarified extracts.

3.2.4. Pretreatment of cell-free extracts and cell lysates

The cell lysate or cell-free extract obtained after sonication in optimum extraction buffer was subjected to three different primary recovery methods, i.e. ammonium sulfate precipitation, acidic precipitation and polymer flocculation/precipitation and each was compared to no pretreatment from the same batch. Each experimental study was performed in replicates.

Ammonium sulfate (Sigma Aldrich Company, USA) precipitation was performed by adding the calculated amount of 3.6 M ammonium sulfate stock solution to the *C. reinhardtii* cell lysates or cell-free extracts to achieve the desired molar concentration (0.5M, 1.0M, 1.5M, 2 M). The lysates or cell-free extracts were then mixed (end over end) for 30 minutes at room temperature followed by centrifugation at 10000 x g for 10

min. The supernatants were filtered with PES 0.45 μm syringe filter and analyzed for host cell protein (HCP), DNA, chlorophyll, turbidity, and αCD22 scFv by methods discussed below.

Acidic precipitation was performed by adding 0.5 N HCl (dropwise with gentle shaking) to *C. reinhardtii* cell lysates or cell-free extracts until the pH dropped to 4.5. Agitation was provided by end-over–end gentle mixing for 15 min, followed by centrifugation at 10000 x g for 10 min. The acidic supernatants were adjusted to pH 8 with 1 N NaOH. The supernatants were filtered with PES 0.45 μm syringe filter and analyzed for host cell protein (HCP), DNA, chlorophyll, turbidity, and αCD22 scFv by methods discussed below.

Polymer pretreatment was done with 10 mg/mL stock solution of chitosan in 1% acetic acid solution (Sigma Aldrich Company, USA). The stock solution of chitosan was prepared by mixing chitosan in 1% acetic acid solution for 24 h at 60 rpm and room temperature. Thirty milligrams of chitosan (3 ml stock) was added per gram of algae biomass followed by vortexing for 30 sec. The lysate pH was then adjusted to 5.0 with 0.1 N HCl and mixed (end over end) for 30 minutes at room temperature. The cell lysate was then readjustment to pH 8.0 and centrifuged at 6000 x g for 10 min for precipitating out chitosan and chitosan bound debris and impurities from the extract. The supernatants were filtered with PES 0.45 μm syringe filter and analyzed for host cell protein (HCP), DNA, chlorophyll, turbidity, and αCD22 scFv by methods discussed below.

3.2.5. Protein analysis

Filtered algal crude extract and purified samples were analyzed by SDS-PAGE, Western blot, and total eluted protein determined by Bradford assay [97]. Host cell protein/Total soluble protein from crude extract and purified samples were quantified using the microplate protocol (working range from 1 to 25 µg/mL and 25 to 1500 µg/mL) Coomassie plus [97] assay kit (Thermo Scientific). Absorption at 595 nm was measured using the VERSA max microplate reader.

NuPAGE Novex Bis-Tris pre-cast gradient gels (4 - 12 %) from Invitrogen™ (1.5 mm x 10 wells), (Cat no. NP0335BOX) were used for SDS-PAGE electrophoresis. Reducing buffer was prepared using LDS sample buffer (4X) (NuPAGE NP0007) containing 10% of reducing agent (Cat no. NP0004). Reduced samples were prepared using a 1:4 ratio reducing-buffer: sample and heated at 70 ºC for10 min. MES SDS Running Buffer (20X) (Cat no. NP0002) stock solution was used to prepare 1X running buffer in RO water. Antioxidant (NuPAGE NP0005) was added to ensure reduced samples during electrophoresis. Gels were run for 35 min at constant voltage (200 V). For SDS analysis, the gels were stained in Coomassie™ G-250 stain (Cat no. LC6065) for 3 hours followed by destaining in RO Water. For Western blot analysis, the gel was transferred to nitrocellulose membranes using iBlot® 7-Minute Blotting System, Life Technologies Corporation.

After protein transfer to a nitrocellulose membrane, the membrane (free sites) was blocked with 2.5 % non-fat milk in TBS containing 0.05 % Tween 20 at pH 7.5 buffer for 1 h to prevent nonspecific binding of the detection antibodies. FLAG-tagged

recombinant proteins (αCD22 scFv and Pfs25) were detected by using anti-FLAG M2-AP (alkaline phosphatase conjugated) antibody from Sigma Aldrich [40 A9469] at a concentration of 1:1,000. After incubation with the antibody for 1 h, the membrane was washed with TBS containing 0.05 % Tween 20 at pH 7.5, buffer and blots were visualized (developed) with nitro-blue tetrazolium (NBT) and 5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt (BCIP) (Sigma FAST B5655) dissolved in 10 mL of filtered RO water.

3.2.6. FLAG affinity chromatography

Crude extracts were filtered using polyethersulfone (PES) 0.45 µm filter and mixed with anti-FLAG affinity resin (Sigma Aldrich A4596) equilibrated in the same lysis buffer used for protein extraction. Approximately 1 mL of resin was used per every 4 g of wet algal biomass. Binding of the recombinant protein to the affinity resin was performed for 2 h at 4 ºC by continuous end-over-end mixing in a Glass Col rotor at ~ 33 rpm (40 % speed control). Affinity resin was washed with 10 column volumes (CV) of lysis buffer followed by 3 column volume of lysis buffer without Tween. The washed FLAG resin was transferred into Bio Spin disposable chromatography columns (Bio Rad, Cat # 732-6008) for protein elution at room temperature. Recombinant protein was eluted at pH 3.5 using 5 CV of 100 mM glycine buffer, pH 3.5 that contained 400 mM NaCl. Eluted protein fractions were collected in 5 tubes containing a predetermined amount of 1M Tris-HCl, pH 8.0 to immediately increase the pH of eluted protein to pH 8.0 and avoid protein denaturation. Typically, three elution fractions (E2

to E4) were used for estimation of purity and yield, although some losses were occurring by not taking into account E1 (Elution fraction 1). By pooling these three fractions, more than 80% of extracted FLAG-tagged proteins were recovered. Extraction buffer and all the materials used including the sonication probe (1/8' microtip A3-561 BRANSON, USA) were cooled in advance.

The FLAG affinity purification method was used as a convenient analytical tool to determine the recombinant extraction yield. The resin was added in sufficient amounts to bind all available FLAG fusion protein present in clarified extracts. Cell debris and supernatants at the end of the batch adsorption period were regularly analyzed by western blotting to assure complete extraction and adsorption, respectively. Although minor recombinant protein losses have occurred during resin washing and pH 3.5 elution from the anti-FLAG resin, this determination of recombinant protein concentration was considered appropriate for estimating recombinant protein in crude extracts.

3.2.7. DNA determination

The DNA concentration in the algae extract subjected to different extraction conditions was determined using a Quant-iT™ PicoGreen ® dsDNA Reagent Kit from Molecular Probes (Eugene, OR). The assay was performed using a fluorescence plate reader, Spectra Max Gemini XS, from Molecular Devices following the procedure recommended by the manufacturer. The samples were excited at 480 nm, and the fluorescent emission intensity at 520 nm was measured. The assay limit is 250 pg/mL.

Standard curve (Range 1-1000 ng/ml) was made using lamda DNA standard present in the kit.

3.2.8. Chlorophyll determination

Chlorophyll (a and b) was measured by measuring the optical density at 652 nm wavelength using a Beckman Coulter (Brea, CA) DU640 spectrophotometer. Lysis buffer (with/without ammonium sulfate; chitosan) was used as blank.

3.2.9. Turbidity measurement

It was measured by measuring the optical density at 750 nm wavelength using a Beckman Coulter (Brea, CA) DU640 spectrophotometer. Lysis buffer (with/without ammonium sulfate; chitosan) was used as blank.

3.2.10. Statistical analysis

Design Expert software was used for the experimental design and analysis. The statistical significance of the models was evaluated by the analysis of variance (ANOVA). Effects with more than 95% of significance (95% confidence interval) that is, effects with a p-value lower than 0.05 were significant. Significantly different means ($P < 0.05$) were separated by the Tukey's test.

3.3. Results and Discussion

3.3.1. Extraction optimization of αCD22 scFv

Previously, we have established that the optimization of algal growth and induced-expression conditions could significantly enhance recombinant protein concentration per gram wet algal biomass [111]. Another way to further enrich algae extracts in recombinant protein concentration is to examine the effect of key extraction variables on recombinant protein yield and the concentration of host cell impurities. A 2^3 full factorial design was performed to determine the effect of salt NaCl, detergent Tween 20, and pH on culture concentration of recombinant protein αCD22 scFv. A biomass-to-buffer ratio of 1:5 (instead of 1:10) was used to reduce the process volume without compromising the amount of extracted recombinant protein. Table 2 compares the effect of different extraction conditions on the concentration of chlorophyll, host cell protein, DNA, and αCD22 scFv in clarified extracts. Each factor was studied on two levels: pH (4.5 and 8), NaCl (100 mM and 400 mM) and Tween (0% and 0.5%). Based on previous extraction data of recombinant proteins from transgenic plant tissues and algae [5, 6, 69, 105], the specific levels of all three factors were chosen. The concentrations of measured impurities and recombinant protein were normalized against those obtained at pH 8.0, 0.4 M NaCl, and 0.5% Tween. At pH 8, both Tween and 400 mM salt were required for maximum extraction of αCD22 scFv. Omitting Tween from the extraction buffer reduced the concentration of both αCD22 scFv and HCP by 29% and 22%, respectively. Chlorophyll concentration at pH 8 was affected (25% reduction) only when Tween was

removed from the buffer and salt concentration reduced to 100 mM. The latter two concentrations further reduced the extracted HCP and αCD22 scFv by about 20%. DNA concentration at pH 8 was not affected by any of tested variables. DNA concentration was significantly reduced (by 40 %) only at pH 4.5 with no Tween and 100 mM salt. αCD22 scFv concentration remained at about 70% for all pH 4.5 tested conditions. At pH 4.5, Tween alone had a pronounced effect on extracted chlorophyll at both salt concentrations, reduction by 45% and 60%. HCP was reduced from 62% to 22% in the absence of Tween if pH 4.5 buffer contained 400 mM salt. It was thus observed that buffer of high pH with high salt and Tween maximize host cell protein extraction, whereas pH 4.5 and low or no salt in the extraction buffer results in minimum amount of host cell proteins. In conclusion, a combination of low pH buffer, low ionic strength (low salt concentration) and absence of Tween in the lysis buffer could significantly reduce chlorophyll (70%) and total soluble protein (55%) concentrations; unfortunately, under those conditions a loss of 30% αCD22 scFv was also observed. Chloroplast expressed proteins may require detergents Tween to reduce hydrophobic interaction with thylakoid membranes similarly to recombinant proteins expressed in tobacco chloroplast [70, 71], but this effect has been observed at pH 8 and not pH 4.5. Our data suggest that the recovery of chloroplast-expressed protein is somewhat related to the solubilization of host-cell protein and probably to reduction of hydrophobic protein-protein and protean–membrane interactions. The maximum recovery yield of αCD22 scFv was achieved at pH 8 with 400 mM salt and 0.5% Tween, the conditions that also maximize HCP, DNA, and chlorophyll extractability. Adjusting the pH of the algae

lysate to <5.0 reduces solubility of most abundant protein (RubisCo), which has a pI of 5.2 and indirectly chlorophyll pigments attached to host cell protein. The addition of Tween at pH 4.5 increased chlorophyll solubility and host cell protein, but did not result in measureable change of αCD22 scFv. The extraction data suggest that pH 4.5, 100 mM, and no Tween conditions are optimal with respect to extract impurities and potentially interesting if 70% αCD22 scFv extraction yield would be acceptable.

Table 2. Effect of extraction conditions (pH, NaCl, Tween) on normalized concentrations of chlorophyll, HCP, αCD22 scFv and DNA. Values given are averages from 3 replicates. [a,b] For each observation, means within a column which are not followed by a common superscript letter are significantly different ($P < 0.05$). [x] Standard deviation.

Extraction conditions	Criteria studied			
pH, NaCl (mM),Tween %	Chlorophyll (%)	HCP (%)	αCD22 scFv (%)	DNA (%)
8, 400, 0.5	100 [a] ± 6.7 [x]	100 [a] ± 13.4	100 [a] ± 14.6	100 [a] ± 10.4
8, 400, 0	98.3 [a] ± 0.5	78.3 [b] ±16.3	70.9 [b] ± 1.9	115.5 [a] ± 2.2
8, 100, 0.5	95.8 [a] ± 3.9	103 [a] ± 4.0	52.4 [b] ± 19.5	105.9 [a] ± 8.7
8, 100, 0	75 [b] ± 3.5	56 [b] ± 15.5	62.5 [b] ± 15.6	135 [a] ± 18.1
4.5, 400, 0.5	73.5 [b] ± 4.9	61.5 [b] ± 9.9	62 [b] ± 24	128.3 [a] ± 25
4.5, 400, 0	28.5 [c] ± 2	21.6 [c] ± 8.3	74 [b] ± 4.2	92.2 [a] ± 8.3
4.5, 100, 0.5	77.8 [b] ± 4	50 [b] ± 15.3	70 [b] ± 21.2	98.2 [a] ± 7.2
4.5, 100, 0	17.9 [d] ± 0.9	45.7 [b] ± 0.9	71.1 [b] ± 5.5	61.9 [b] ± 4.2

3.3.2. Evaluation of different primary recovery methods

Optimum extraction conditions that are critical for maximum product recovery are often optimal for the release of a variety of host cell proteins and other water soluble cell components such as DNA, chlorophyll pigments, polysaccharides, and proteases. Since these components can affect the final product quality and reduce purification it is important to condition and pre-treat the extract before further purification. After homogenization, the cell lysate is commonly subjected to solid-liquid separation by centrifugation to remove the cell debris and insoluble impurities to form cell-free extract (Figure 7). As shown in Figure 7, pretreatment methods can be implemented either on cell-free extract or directly on the cell lysate. Direct pretreatment of cell lysate, whenever possible, is desirable because a single centrifugation step could be used to remove cell derris and other precipitated or flocculated lysate impurities. The choice to pretreat cell lysate or cell-free extracts will made by determining recombinant protein yield after selected pretreatment and overall pretreatment efficacy i.e. removal of undesirable impurities by the end of primary recovery process (Figure 7).

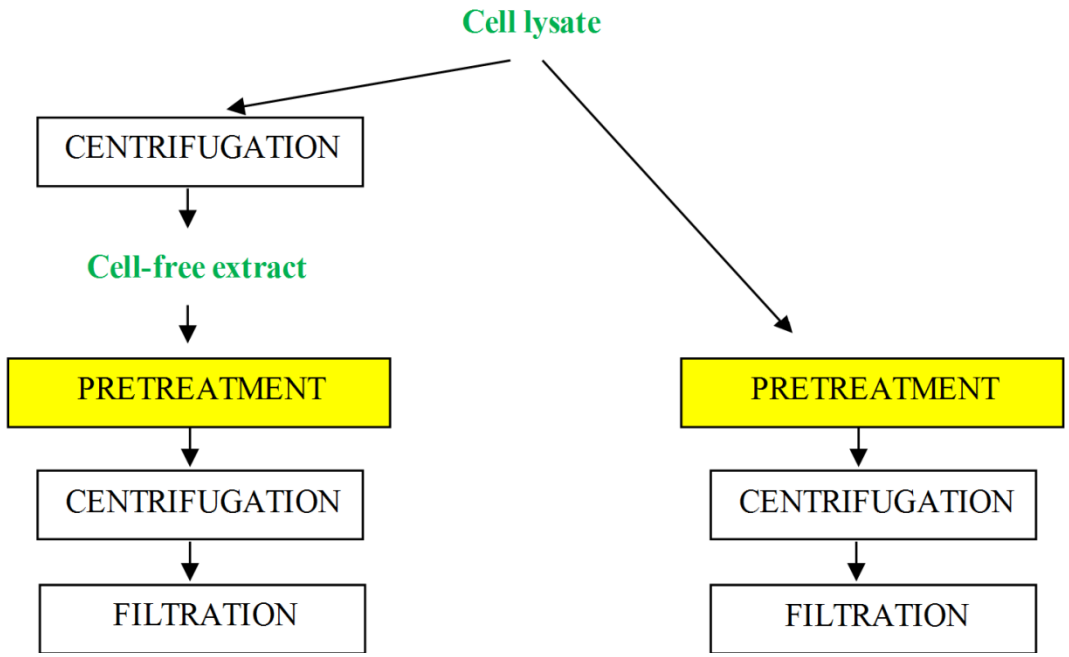

Figure 7. Primary recovery steps of αCD22 scFv from *C. reinhardtii* lysate.

After homogenization of *C. reinhardtii* cells in the optimum extraction buffer, different pretreatments were evaluated for clarification of algae extract from cell-free extract and cell lysate. The choice of these pretreatment methods mainly depended on factors like compatibility of the method with further affinity purification steps, product quality and yield and reduction of overall impurity burden. Different primary recovery methods evaluated were ammonium sulfate precipitation, acidic precipitation, and polymer precipitation.

The preliminary screening of different concentration of ammonium sulfate was done to obtain maximum reduction of host cell protein and chlorophyll in the algae extract. The plotting trend in Figure 8 shows the effect ammonium sulfate concentration on host cell protein and chlorophyll remaining in the algae extract after precipitation. It

was observed that increasing the concentration of ammonium sulfate in the algae extract resulted in more reduction of proteins and chlorophyll in the supernatant after precipitation. Ammonium sulfate concentration of (1 M) or more had a notable effect on the reduction of host cell protein and chlorophyll in the extract. A proportional decrease in the chlorophyll pigments can be attributed to pigments attachment to precipitated proteins. By following a conservative approach to prevent the loss of the recombinant protein in the pellet, 1M concentration was chosen for further study. Also, with higher saturation there is a possibility of interference from ammonium sulfate in subsequent affinity chromatography.

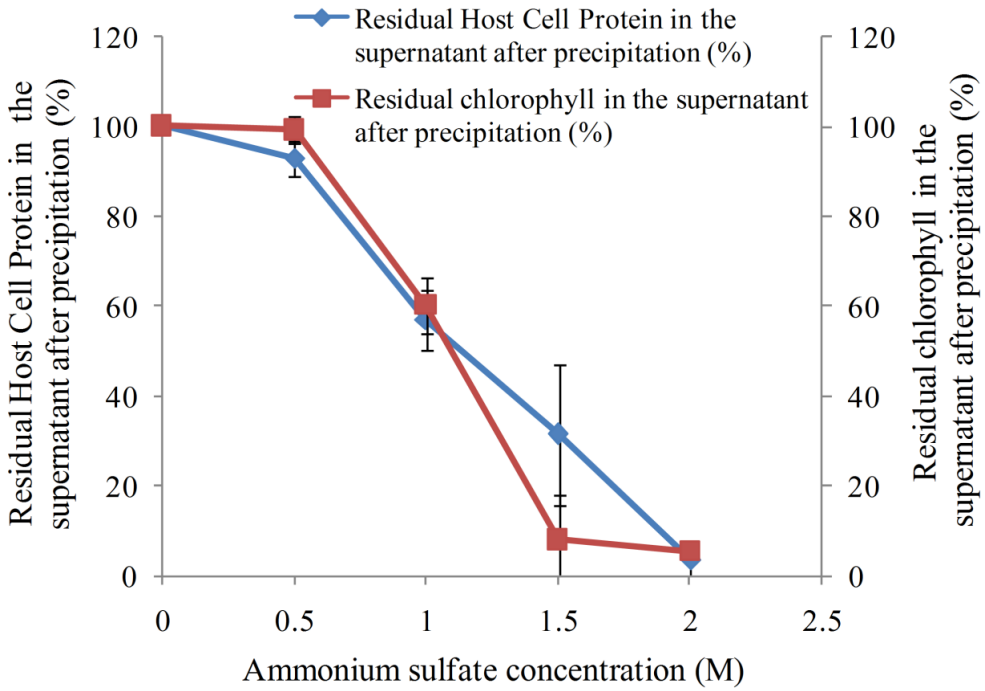

Figure 8. Effect of ammonium sulfate concentration on residual host cell protein (HCP)and chlorophyll in supernatants after precipitation. Values plotted are averages from 3 replicates; bars represent standard deviation.

The effect of different primary recovery methods on the conditioning of algae extract is summarized in Table 3 and Table 4. Ammonium sulfate precipitation and acidic precipitation was done on cell-free extract and cell lysate, whereas polymer precipitation with chitosan at pH 5 was done on cell lysate. The clarification of algae extract after each pretreatment was evaluated by quantification of impurities, namely DNA, chlorophyll pigments, host cell proteins, aggregates (turbidity) and recovery percentage of target recombinant protein αCD22 scFv measured by anti-FLAG affinity chromatography. Control experiment (no pretreatment) was performed from the same algae batch to minimize the effect of batch-to-batch variability. Each criterion was compared and normalized to the respective concentrations obtained with the control. In comparison to the control, the host cell protein of the pretreated extracts (acidic precipitation and ammonium sulfate precipitation) of both cell-free lysates and cell lysates was reduced to a large extent (31%-57%), but did not vary significantly with the pretreatment type, i.e. acid vs. salt precipitation. In contrast, the chlorophyll content showed a wide range among the pretreatment tested and was reduced by 30% (cell-free lysate) to 70% (cell lysate) in ammonium sulfate precipitation and 72% (cell-free lysate) to 45% (cell lysate) in case of acidic precipitation. However, both the pretreatments failed to reduce the DNA content. In both, ammonium sulfate precipitation and acidic precipitation, it is evident that there is a significant difference in the yield of αCD22 scFv and chlorophyll between cell-free extract and cell lysate. When cell free extract was subjected to acidic precipitation, a αCD22 scFv yield of 118.8% ± 21.8 was reported, compared to a yield of 87.5% ± 5.06 in case of ammonium sulfate

precipitation. The yield of αCD22 scFv from pretreated cell-free extracts was significantly different from that obtained from pretreated cell lysates (73.12% ± 10.9 for acidic and 79.8% ± 10.9 for ammonium sulfate). In the experiments with cell lysates, αCD22 scFv losses ranged from about 20% to 27%. If the given losses are acceptable, the flexibility of operating the two pretreatment methods (acidic precipitation and ammonium sulfate precipitation) on cell lysate would be more economical as it allows cell debris removal and impurity reduction simultaneously with only one centrifugation step.

An interesting observation was made when we compared acidic precipitation at pH 4.5 of cell lysate (50 mM Tris, 400 mM NaCl, 0.5% Tween) with the extraction at pH 4.5 in 50 mM Tris, 400 mM NaCl, 0.5% Tween. In the former, there was a reduction in αCD22 scFv yield by about 27%, whereas during the extraction under the same conditions we observed a significantly greater loss of 38%, probably due to the lower solubility of acidic host proteins. The reduction αCD22 scFv extraction yield by 38% was the similar to that of HCP (39%).

In contrast to acidic and ammonium sulfate precipitation, the lysate pretreatment with chitosan at pH 5 proved to be a better method in terms of impurities reduction in the algae extract (Table 4). It not only reduced the chlorophyll (58%) and protein (82%), but chitosan was able to flocculate the cell debris making their removal with low speed centrifugation possible. Most importantly, there was a significant reduction in DNA content to 14%, resulting in a less viscous and easier to filter extracts. However, the polymer pretreatment methods led to a 35% loss of αCD22 scFv, when compared to the

control. This loss is relatively high for a primary recovery steps, but because of the other benefits further optimization of this pretreatment method is probably warranted.

Table 3. Effect of pretreatment method of cell-free extract on residual DNA, chlorophyll, host cell protein, and αCD22 scFv yield in algae extract. Values given are averages from 3 replicates ± standard deviations. [a,b,c] For each observation, means which do not share a common superscript letter are significantly different from the control no pretreatment ($P < 0.05$).

	Residual DNA (%)	Residual Chlorophyll (%)	Residual Host Cell Protein (%)	αCD22 scFv yield (%)
No Pretreatment	$100^{a} \pm 9.12$	$100^{a} \pm 4.8$	$100^{a} \pm 14.5$	$100^{a} \pm 16.3$
Ammonium sulfate precipitation at pH 8	$100^{a} \pm 1.3$	$60.1^{b} \pm 6.3$	$56.7^{b} \pm 6.7$	$87.5^{a} \pm 5.06$
Acidic precipitation at pH=4.5	$91.7^{a} \pm 7.6$	$28.4^{c} \pm 14.7$	$31.1^{c} \pm 9.3$	$118.8^{a} \pm 21.8$

Table 4. Effect of pretreatment method of cell lysate on residual DNA, chlorophyll, host cell protein, and αCD22 scFv yield in algae extract. Values given are averages from 3 replicates ± standard deviations. [a,b,c] For each observation, means which do not share a common superscript letter are significantly different from the control ($P < 0.05$).

	Residual DNA (%)	Residual Chlorophyll (%)	Residual Host Cell Protein (%)	αCD22 scFv yield (%)
No Pretreatment	$100^{a} \pm 9.12$	$100^{a} \pm 4.8$	$100^{a} \pm 14.5$	$100^{a} \pm 16.3$
Ammonium sulfate precipitation at pH 8	$100^{a} \pm 0.1$	$31.5^{b} \pm 8.2$	$46.1^{b} \pm 7.4$	$79.8^{b} \pm 10.9$
Acidic precipitation at pH=4.5	$92.6^{a} \pm 10.5$	$55^{c} \pm 1.6$	$36.1^{b} \pm 1.6$	$73.12^{bc} \pm 10.9$
Chitosan precipitation at pH=5.0	$14^{b} \pm 1.41$	$42.4^{b} \pm 0.2$	$17.7^{c} \pm 4.9$	$65.3^{c} \pm 1.72$

All three pretreatment methods were able to decrease turbidity of centrifuged extracts (Figure 9). Ammonium sulfate and acidic precipitation methods decreased turbidity of lysates and cell-free extracts from 0.49 ± 0.08 to 0.15 ± 0.03 and 0.27 ± 0.11, respectively. The chitosan-mediated pretreatment resulted in more than tenfold decrease in extract turbidity. Lower viscosity of chitosan treated extracts along with the significant decrease in turbidity (OD_{750} = 0.04) affected 0.45 µm filtration efficiency significantly. We have observed a threefold increase in filtration throughput (processed volume / area) with chitosan pretreatment, compared to 1.5 fold each with ammonium sulfate and acidic precipitation.

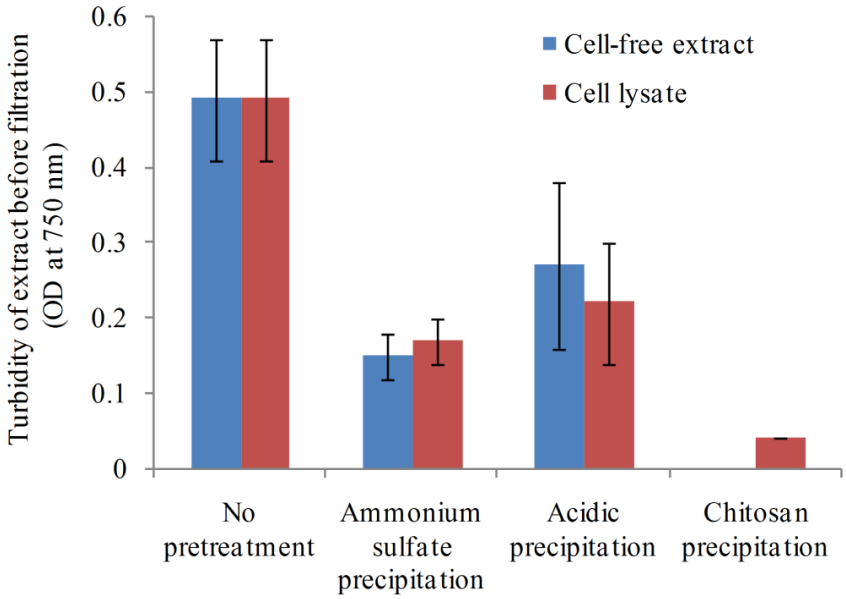

Figure 9. Effect of different pretreatment methods on turbidity ($O.D._{750}$) of algae extract after centrifugation and before 0.45 µm filtration. Values plotted are averages from 3 replicates (bars are standard deviations).

All pretreatment methods tested in this work have pros and cons, which depend on the target protein to be purified and compatibility with potential capture (first purification) methods. For instance, if polymer flocculation is being considered for clarification, diligent consideration must be made to verify the impact of polymer on the binding capacity of affinity chromatography as well as ensuring removal of the flocculating agents to acceptable levels in the drug product. For ion exchange capture step, it is preferable not to alter the ionic strength of the extract during pretreatment to avoid the conductivity adjustment by dilution or diafiltration before loading onto the capture column. Thus, prior to ion exchange chromatography, a salt induced precipitated extract will likely require dilution or diafiltration step. For example, incase of clarification of rice cell suspension containing HSA, dialysis was done after 40% ammonium sulfate precipitation prior to loading on anion exchange column [76]. Alternatively, to skip the dilution or removal of ammonium sulfate from clarified extracts after the precipitation step, one could choose to use hydrophobic interaction chromatography as a capture step, as in case of tobacco cell suspension extract containing GFP-fusion protein [75]. After acidic precipitation, additional pH adjustment of the extract may be required for efficient binding of the target protein to an affinity, ion exchange, or HIC resin. Acidic precipitation, otherwise, does not significantly alter extract conductivity. Also, the pH and net protein charge dictates the selection between anion and cation exchange chromatography. Thus, pretreatment methods should be chosen on a case-by-case basis and modified accordingly. The testing of these primary

recovery methods on microalgae *C. reinhardtii* extracts certainly lays down a road map for further downstream processing testing for algae platform.

3.3.3. Analysis of pretreated extracts by FLAG affinity chromatography

After being subjected to different extraction and primary recovery method, recombinant protein was purified from clarified crude lysates by FLAG affinity adsorption and analyzed by Western blotting. In each experimental study, αCD22 scFv protein purified from filtered algae extract (from the same batch) without pretreatment was used as control.

Figure 10 demonstrates the comparison of the eluted fractions of αCD22 scFv purified from ammonium sulfate treated cell-free algae extract vs. no pretreatment. The presence of bands of negligible intensity in the supernatant after 2 hours of incubation (lane 7) at the same size as αCD22 scFv (30 kDa) confirm the efficient binding of FLAG-tagged αCD22 scFv to the anti-FLAG affinity resin in presence of ammonium sulfate. In both control and pretreated extracts, total eluted protein analysis by Bradford, indicated that the majority of purified αCD22 scFv (30 kDa band) eluted in fractions 2, 3 and 4 (lanes 2,3 and 4; lanes 8, 9 and 10). These three fractions typically contained approximately 80% of the total eluted recombinant protein from the resin. Western blot revealed the presence of minor degradation products of 14 and 17 kDa in size. The analyses of purified αCD22 scFv from pretreated extracts (lanes 8, 9 and 10) revealed no significant deviation in quality compared to the eluted fractions (lanes 2, 3 and 4) from control (no pretreatment) extract. Thus, ammonium sulfate precipitation did not affect

the further downstream affinity chromatography or the quality and yield of αCD22 scFv (87.5 % ± 5.06 for cell-free extract) adversely. However, further analysis is required to confirm the same.

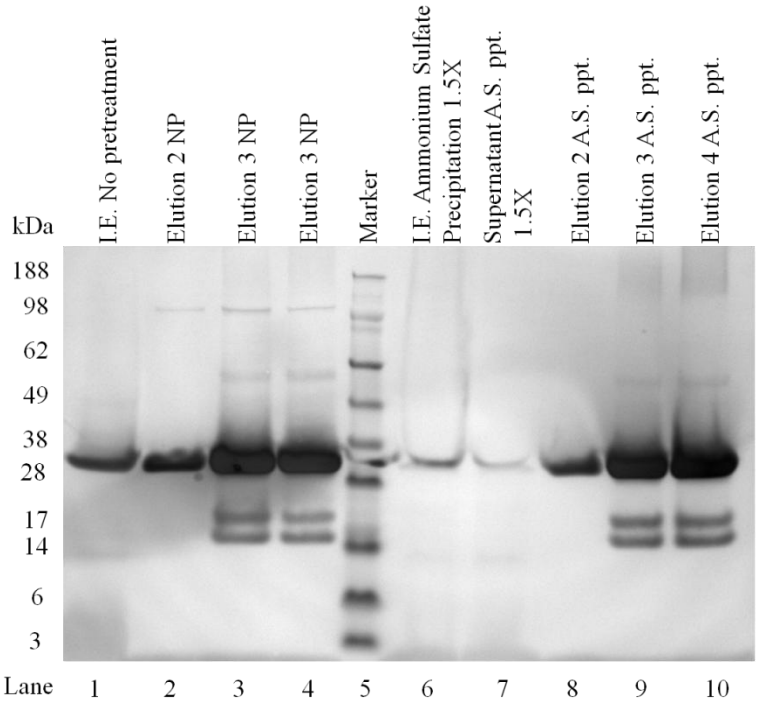

Figure 10. Comparison of Western blot analysis (using anti-FLAG-AP conjugated antibody) of *C. reinhardtii* αCD22 scFv purified from cell-free extract treated with ammonium sulfate precipitation vs. no pretreatment control. Lane 1: clarified initial extract (no pretreatment); Lanes 2 to 4: pH 3.5 eluted fractions (no pretreatment); Lane 5: molecular weight marker (kDa); Lane 6: clarified initial extract after ammonium sulfate precipitation (1.5 times diluted); Lane 7: 1.5 times diluted supernatant after 2 h of binding with anti-FLAG resin (ammonium sulfate precipitation); Lanes 8 to 10: pH 3.5 eluted fractions (ammonium sulfate precipitation).

The comparison of the eluted fractions of αCD22 scFv purified from acidic precipitation treated cell-free extract with no pretreatment is shown in Figure 11. The

analyses of purified αCD22 scFv from pretreated extracts (lanes 1, 3, 4 and 5) revealed no significant degradation when compared to the eluted fractions (lanes 7, 8, 9 and 10) from control (no pretreatment) extract. αCD22 scFv seems to remain stable at low pH for a limited amount of time tested. However, further analysis is required to confirm the same.

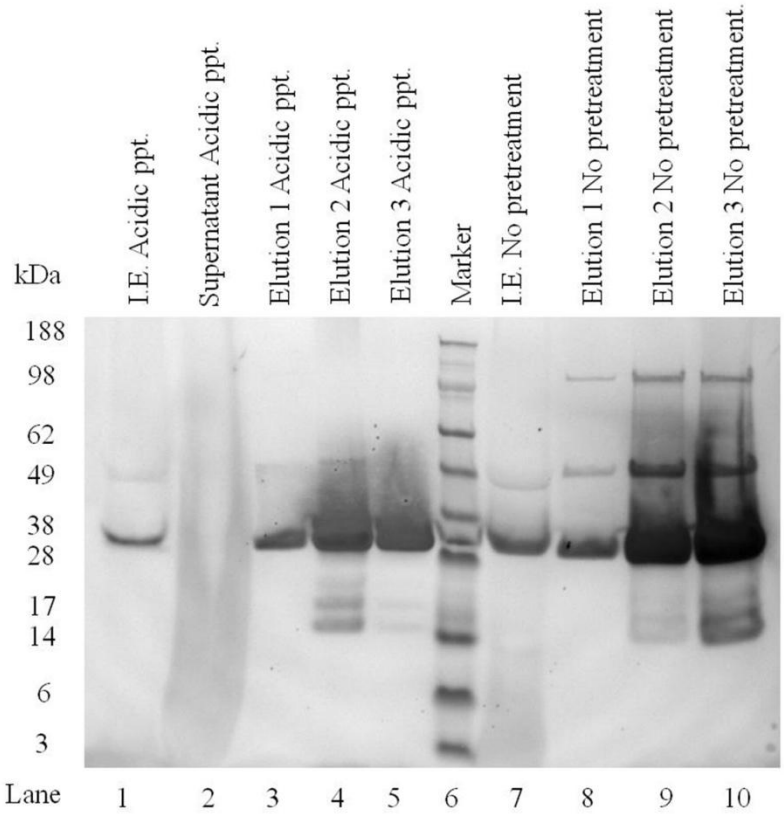

Figure 11. Comparison of Western blot analysis (using anti-FLAG-AP conjugated antibody) of *C. reinhardtii* αCD22 scFv purified from cell-free extract treated with acidic precipitation vs. no pretreatment control. Lane 1: clarified initial extract after acidic precipitation; Lane 2: Supernatant after 2 h of binding with anti-FLAG resin (acidic precipitation); Lane 3 to 5: pH 3.5 eluted fractions (acidic precipitation); Lane 6: molecular weight marker (kDa); Lane 7: clarified initial extract (no pretreatment); Lanes 8 to 10: pH 3.5 eluted fractions (no pretreatment).

As discussed above, with chitosan precipitation at pH 5, we were able to reduce most of the impurities including DNA, proteins, chlorophyll and debris. However, we also observed a significant loss (35%) of the target protein, αCD22 scFv. This was also prevalent in the western blot analysis (Figure 12) of eluted fractions and supernatant after 2 h of binding with Anti FLAG resin (chitosan precipitation). In case of chitosan precipitation, there was a high intensity band of 30 kDa in the supernatant after 2 h of binding to anti-FLAG affinity resin, suggesting possible interference with binding of FLAG tagged recombinant protein to the anti-FLAG resin in presence of chitosan. However, a slight band of 30kDa was also observed in supernatant of no pretreatment (lane 2) suggesting inefficient binding with anti-FLAG resin. The results are thus inconclusive. Polymer precipitation is promising but it needs further optimization to prevent loss of αCD22 scFv.

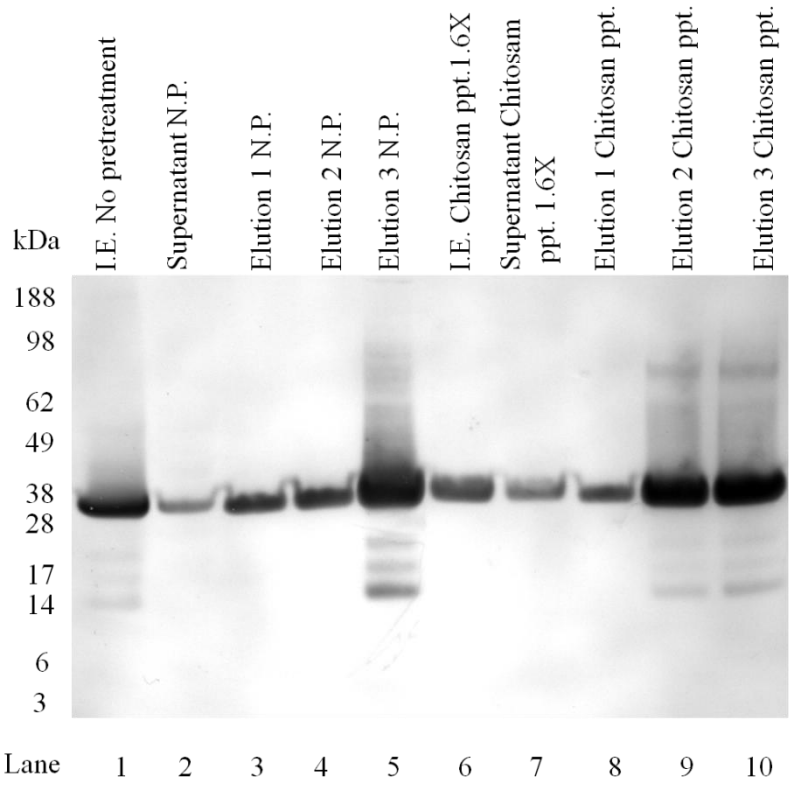

Figure 12. Comparison of Western blot analysis (using anti-FLAG-AP conjugated antibody) of *C. reinhardtii* αCD22 scFv purified from cell lysate treated with chitosan precipitation vs. no pretreatment control. Lane 1: clarified initial extract (no pretreatment control); Lane 2: supernatant after 2 h of binding with anti-FLAG resin (no pretreatment control); Lanes 3 to 5: pH 3.5 eluted fractions (no pretreatment); Lane 6: clarified initial extract after chitosan precipitation (1.6 times diluted); Lane 7: 1.5 times diluted supernatant after 2 h of binding with anti-FLAG resin (chitosan precipitation); Lanes 8 to 10: pH 3.5 eluted fractions (chitosan precipitation).

3.4. Summary

This study investigated the process variables affecting extraction and recovery of recombinant protein in the algae extract. Composition of extraction buffer was optimized based on three factors - pH, NaCl (mM) and Tween. The maximum recovery yield of αCD22 scFv was achieved at pH 8 with 400 mM salt and 0.5% Tween, the conditions

that also maximize host cell protein (HCP), DNA, and chlorophyll extractability. A combination of low pH buffer, low ionic strength (low salt concentration) and absence of Tween in the lysis buffer could significantly reduce chlorophyll (~70%) and total soluble protein (~55%) concentrations; unfortunately, under those conditions a loss of ~30% αCD22 scFv was also observed. In conclusion, along with pH 8, high salt and Tween 20 are required for maximum recovery of chloroplast expressed αCD22 scFv.

It has been identified that a relatively inexpensive upstream protein production platform like by *C. reinhardtii* has to be matched by low cost downstream processing. Investigation of different primary recovery methods, viz., ammonium sulfate precipitation, acidic precipitation and cationic polymer (chitosan) precipitation on cell-free extract as well as cell lysate of *C. renihardtii*, revealed interesting insights. It was interesting to note that both ammonium sulfate precipitation and acidic precipitation performed well with cell-free extract as almost all αCD22 scFv was recovered compared to control with a significant reduction of chlorophyll and HCP. However, with cell lysates, both acidic precipitation and ammonium sulfate precipitation reported a loss of αCD22 scFv by 27% and 20% respectively. Polymer precipitation with chitosan at pH 5 on cell lysate was successful not only in reducing HCP (82%), chlorophyll (58%), debris, but also in remarkably reducing the DNA (hence turbidity) (14%) and turbidity of the algae extract compared to the control. Consequently, this resulted in better filterability of the extract. However, a notable loss of 35% αCD22 scFv was observed with chitosan precipitation. Thus, although polymer precipitation is promising, it needs further optimization to prevent the loss of the target protein.

All the three methods with cell lysate resulted in similar loss of αCD22 scFv yield (~30%). The mechanism of precipitation with the three agents is different. Also, extraction in lysis buffer of pH 4.5 resulted in a αCD22 scFv loss of 38%. This suggests entrapment of αCD22 scFv by precipitated proteins and chitosan formed flocs. It is likely that the target protein gets entrapped in the cell debris while precipitation, resulting in the loss of yield.

According to the western blot analysis, none of the pretreatments altered the quality of the target protein αCD22 scFv. However, further analysis by size exclusion chromatography is required to confirm the same. The selection of these pretreatments for conditioning of algae extract depends on case by case basis. The pretreatment can be selected based on its compatibility with the first downstream purification step or the best pretreatment for a particular target product can guide the selection of the first downstream processing step.

CHAPTER IV

CONCLUSIONS AND RECOMMENDATIONS

4.1. Conclusions

Chlamydomonas reinhardtii chloroplast has a demonstrated potential for producing complex recombinant proteins. This study attempted to investigate the process variables affecting the growth of the microalgae, accumulation and recovery of recombinant protein αCD22 scFv and impurities. This has added to the current scientific knowledge on 'microalgae as biofactories' and will assist in making this platform commercially attractive. The major challenges identified during this study were, i) low transgene expression level, and ii) resin or membrane fouling due to presence of impurities like host cell proteins, DNA, chlorophyll and other pigments.

The problem of low recombinant protein was addressed in first part of the investigation (Chapter II) by optimization the algal cultivation and light inducible accumulation of αCD22 scFv protein. Maximum biomass accumulation of transgenic *C. reinhardtii* was achieved after resuspending the cells in fresh TAP media after five days of heterotrophic growth. Consequently, a 2.8-fold increase in the culture concentration of recombinant therapeutic proteins αCD22 scFv and Pfs25 was observed, but this could also be achieved by adding acetate.

The psbA regulated expression of αCD22 scFv protein in the chloroplast was also significantly affected by light duration and intensity. The exposure of light of 300

μmol m^{-2} s^{-1} for 24 h resulted in a maximum culture concentration of αCD22 scFv (314.0 ± 20 μg/mL), which is a two-fold increase compared to 101 μmol m^{-2} s^{-1}. Thus, an increase in light intensity to 300 μmol m^{-2} s^{-1} not only increased the recombinant protein production, but also led to an efficient product recovery with a two-fold increase of the recombinant protein fraction in the extracted soluble protein. Overall, resuspension and light optimization resulted in a six-fold increase in the recovered recombinant protein in the chloroplasts of *C. reinhardtii*.

The second challenge was dealt in Chapter III. This study investigated the process variables affecting extraction and recovery of recombinant protein and impurities in the algae extract. The maximum recovery yield of αCD22 scFv was achieved at pH 8 with 400 mM salt and 0.5% Tween, the conditions that also maximize host cell protein (HCP), DNA, and chlorophyll extractability. A combination of low pH buffer, low ionic strength (low salt concentration) and absence of Tween in the lysis buffer could significantly reduce chlorophyll (70%) and total soluble protein (55%) concentrations; unfortunately, under those conditions a loss of 30% αCD22 scFv was also observed.

On cell-free extract the two pretreatments (ammonium sulfate precipitation and acidic precipitation) were efficient in reducing chlorophyll and HCP and recovering almost all αCD22 scFv, compared to the control. However, with cell lysates, both acidic precipitation and ammonium sulfate precipitation reported a loss of αCD22 scFv by 27% and 20% respectively. Polymer precipitation with chitosan at pH 5 on cell lysate was promising as it not only resulted in the reducing HCP (82%), chlorophyll (58%), debris,

but also in remarkably reducing the DNA (14%) and turbidity of the algae extract compared to the control and hence resulting in better filterability of the extract. However, a notable loss of 35% αCD22 scFv was observed with chitosan precipitation. Thus, it needs further optimization to prevent the loss of the target protein. All the three methods with cell lysate resulted in similar loss of αCD22 scFv yield (~30%) probably due to entrapment of αCD22scFv in the precipitated proteins and cell debris.

As analyzed by western blots, none of the pretreatments altered the quality of the target protein αCD22 scFv. However, further analysis by size exclusion chromatography is required to confirm the same. The selection of these pretreatments for conditioning of algae extract depends on case by case basis. The pretreatment can be selected based on its compatibility with the first downstream purification step or the best pretreatment for a particular target product can guide the selection of the first downstream processing step.

In both studies, FLAG affinity adsorption was used as an analytical technique for purification and recovery of the recombinant proteins. However, anti-FLAG affinity resin is not sufficiently robust for column scale up. Therefore, other affinity tags or chromatography techniques should be considered to enable robust process purification scale up.

4.2. Recommendations

The suggestions for future work include**:**

1. To investigate the effect of light-dark cycle on the growth of *C. reinhardtii* and accumulation of αCD22 scFv.
2. To further optimize chitosan precipitation with an aim to reduce the loss of αCD22 scFv.
3. To screen different polymers for flocculation of cell debris and reduction of impurities.
4. To screen different affinity and non affinity resins for capture of αCD22 scFv and develop purification process to achieve more than 90% purity.
5. To evaluate the compatibility of different pretreatment methods with the first purification chromatography method.

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APPENDIX

Appendix 1. Effect of resuspension on accumulation of recombinant proteins

Table A1.1. Effect of resuspension on the growth of *C. reinhardtii* and accumulation of recombinant protein αCD22 scFv in the chloroplast.

Cultivation	Biomass (g)	% TSP (µg/µg)	Wet biomass conc. (µg/g)	Culture Conc. of R-protein αCD22 scFv (µg/L)
With resuspension	4	0.12 ± 0.03	35.1 ± 5.8	140.5 ± 23
Without resuspension	2	0.13 ± 0.00	26.3 ± 5.4	52.9 ± 11

Table A1.2. Effect of resuspension on the growth of *C. reinhardtii* and accumulation of recombinant protein αCD22PE40 (MT 47). R: Resuspended; NR: Non resuspended.

Samples	Biomass (g)		Total Soluble Protein (µg/ml)		µg of MT47 recovered in E2-E3		R-protein/TSP (%)		R-protein/wet biomass (%)	
	R	NR	R	NR	R	NR	R	NR	R	NR
Batch 1	3.5	2.3	5973	3243	45.3	11	0.04	0.03	13	4.8
Batch 2	3.9	2.4	3810	3561	14.2	7.2	0.018	0.016	3.7	3
Batch 3	3.7		5459		20.7		0.020		5.6	
Batch 4	3.45		4303		16.5		0.022		4.8	
Batch 5	3.6		4663		22.5		0.027		6.25	
Scale up 36 h light	2.9 g purification		5865		E2-E3: 41.87		0.06		14.5	

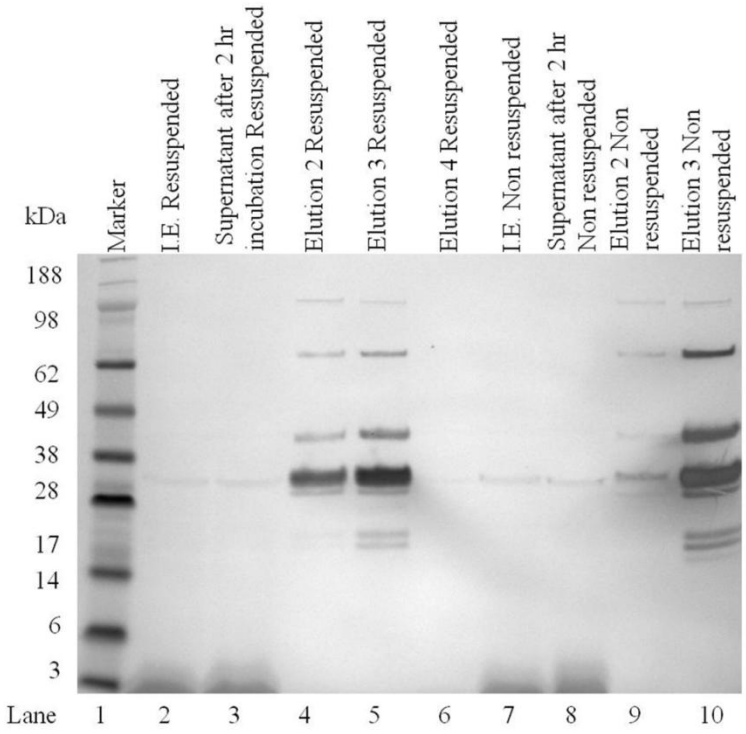

Figure A1.1. Comparison (resuspension vs. non resuspension) of Western blot analysis (using anti-FLAG-AP conjugated antibody) of *C. reinhardtii* containing αCD22PE40 (MT 47) (64kDa) purified from cell-free extract. Lane 1: marker; Lane 2: clarified initial extract (resuspended); Lane 3: supernatant after 2 h of binding with anti-FLAG resin (resuspended); Lanes 4 to 6: pH 3.5 eluted fractions (resuspended); Lane 7: clarified initial extract (non resuspended); Lane 8: supernatant after 2 h of binding with anti-FLAG resin (non resuspended); Lanes 9 to 10: pH 3.5 eluted fractions (non resuspended). Significant degradation was observed.

Appendix 2. Effect of pretreatment on turbidity and filterability of algae extract

Table A2.1. Effect of pretreatment and stage of pretreatment on the turbidity of algae extract and filtration load capacity (0.45 μm).

	Stage of Primary Recovery	Pre-filter OD_{750}	Post-filter OD_{750}	Load Capacity (%)
No Pretreatment	Filtered Extract	0.49 ± 0.08	0.23 ± 0.04	100
Ammonium sulfate (1M) precipitation	Cell free extract	0.15 ± 0.03	0.05 ± 0.04	244 ± 204
	Cell lysate	0.17 ± 0.03	0.07 ± 0.06	165.5 ± 48.7
Acidic precipitation at pH=4.5	Cell free extract	0.27 ± 0.11	0.16 ± 0.04	177 ± 89.1
	Cell lysate	0.22 ± 0.08	0.14 ± 0.03	137 ± 41
Chitosan precipitation	Cell lysate	0.04 ± 0.00	0.02 ± 0.01	263 ± 1.86

Appendix 3. Purification of αCD22 scFv by Capto L affinity chromatography

Table A3.1. Batch purification of αCD22 scFv (purified by anti-FLAG affinity chromatography) on Capto L resin.

αCD22 scFv (FLAG purified)	αCD22 scFv (Capto L purified)	Yield
56.1 µg	23.9 µg	43%

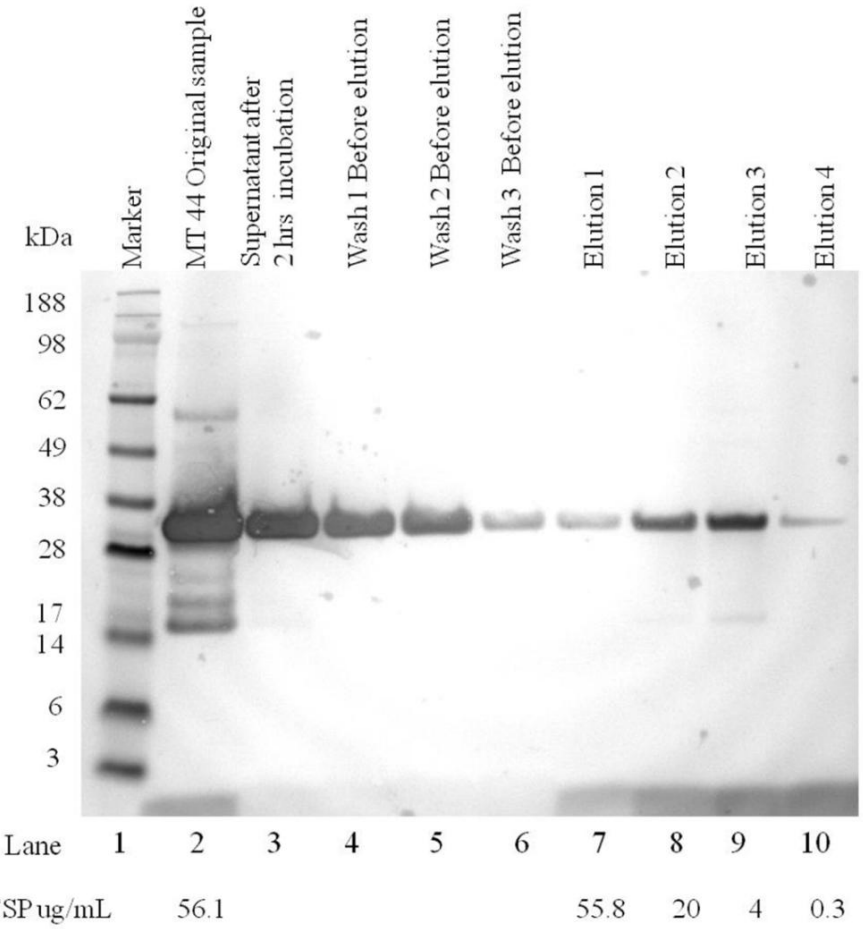

Figure A3.1. Western blot analysis (using anti-FLAG-AP conjugated antibody) of *C. reinhardtii* αCD22 scFv purified from cell-free extract by Capto L resin affinity chromatography. Lane 1: marker; Lane 2: αCD22 scFv (MT44) purified by anti-FLAG resin; Lane 3: supernatant after 2 h of binding with Capto L resin; Lanes 4 to 6: washes before elution; Lanes 7 to 10: pH 3.5 eluted fractions.

Table A3.2. Comparison of batch purification of αCD22 scFv by anti-FLAG and Capto L affinity chromatography.

αCD22 scFv (FLAG purified)	αCD22 scFv (Capto L purified)	Yield by Capto L
25.73 μg/g biomass	13.9 μg/g biomass	54.4%

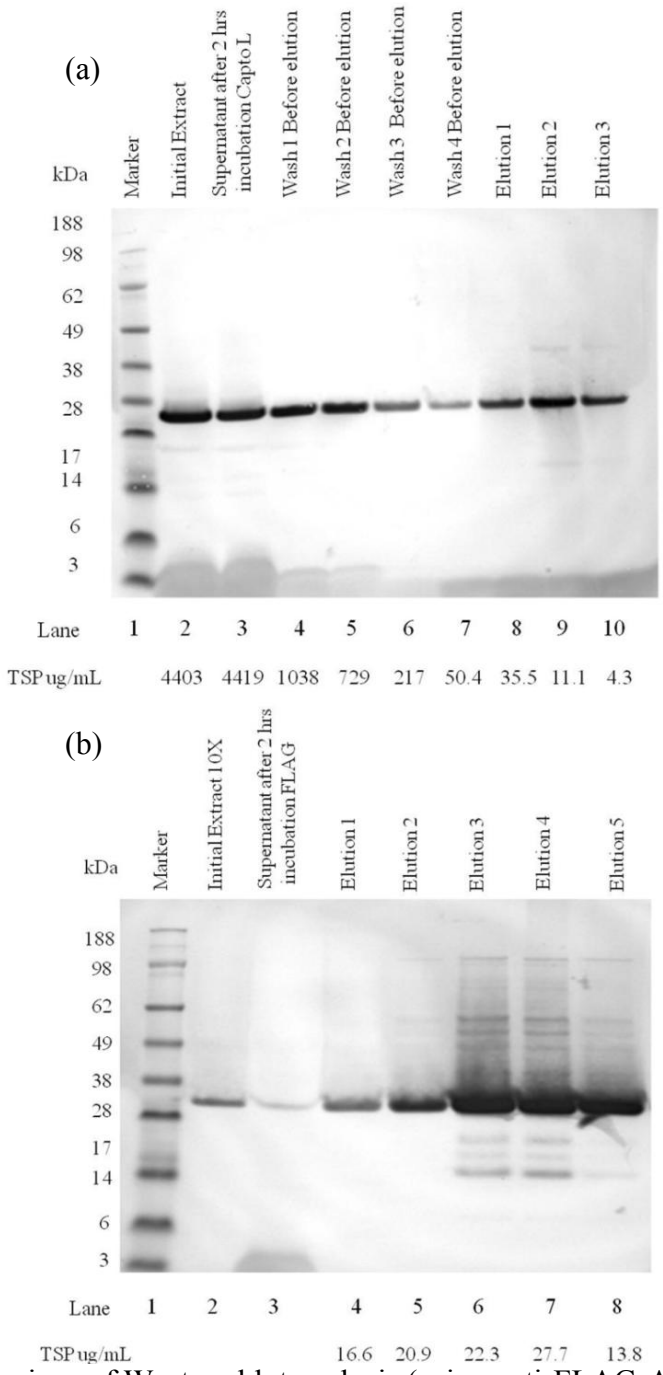

Figure A3.2. Comparison of Western blot analysis (using anti-FLAG-AP conjugated antibody) of *C. reinhardtii* αCD22 scFv purified from cell-free extract by Capto L resin (a) and anti-FLAG resin (b). Lane 1: marker; Lane 2: clarified initial extarct; Lane 3: supernatant after 2 h of binding with Capto L resin and anti-FLAG; Lanes 4 to 7: washes before elution (Capto L resin); Lanes 8 to 10: pH 3.5 eluted fractions (Capto L resin); Lanes 4 to 8: pH 3.5 eluted fractions (anti-FLAG resin).

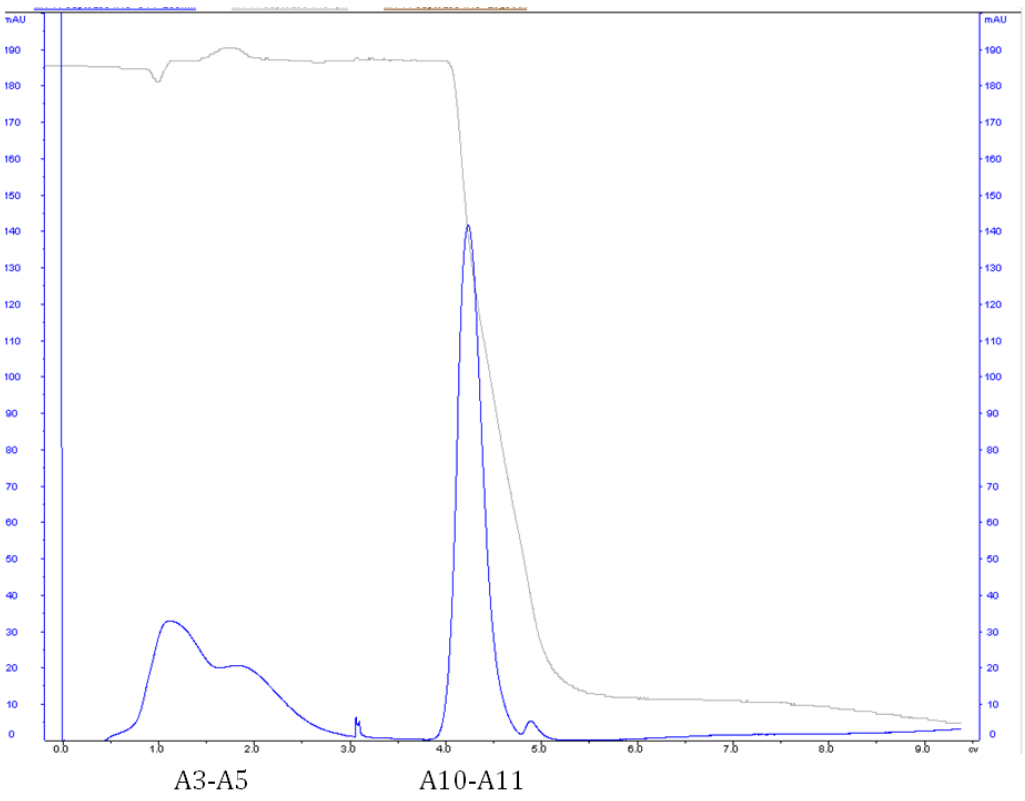

Figure A3.3. Anti-FLAG purified αCD22 scFv loaded on Capto L resin affinity column. UV_{280}: Two peaks at flow through and one peak at elution was observed.

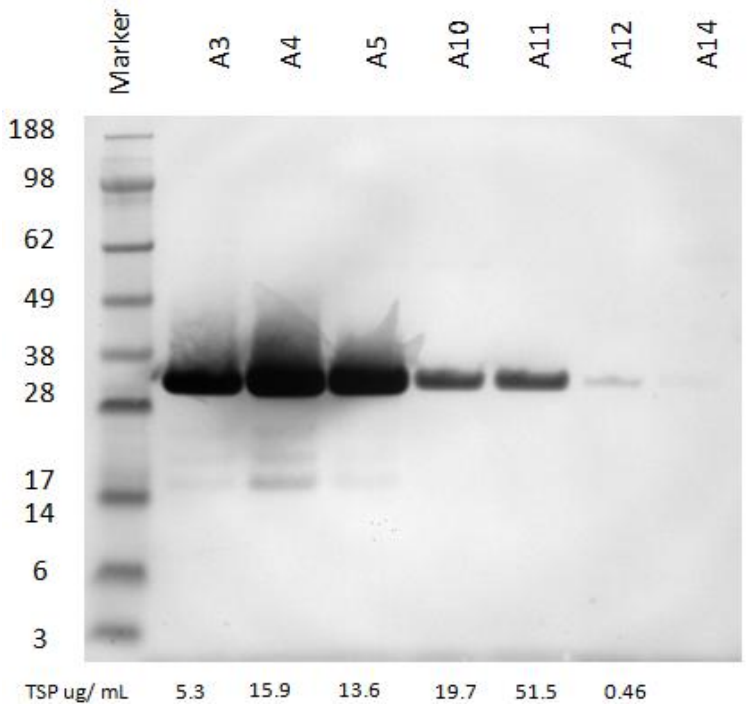

Figure A3.4. Western blot analysis (using anti-FLAG-AP conjugated antibody) of *C. reinhardtii* containing αCD22 scFv purified from Capto L Affinity column (chromatogram in Figure A3.3); Lane 1: marker; Lane 2 to 4: flow through fractions A3-A5; Lane 5 to 8: elution fractions A10-A14. There was significant loss of αCD22 scFv in the flow through. Hence Capto L resin affinity chromatography was inefficient for the target protein.

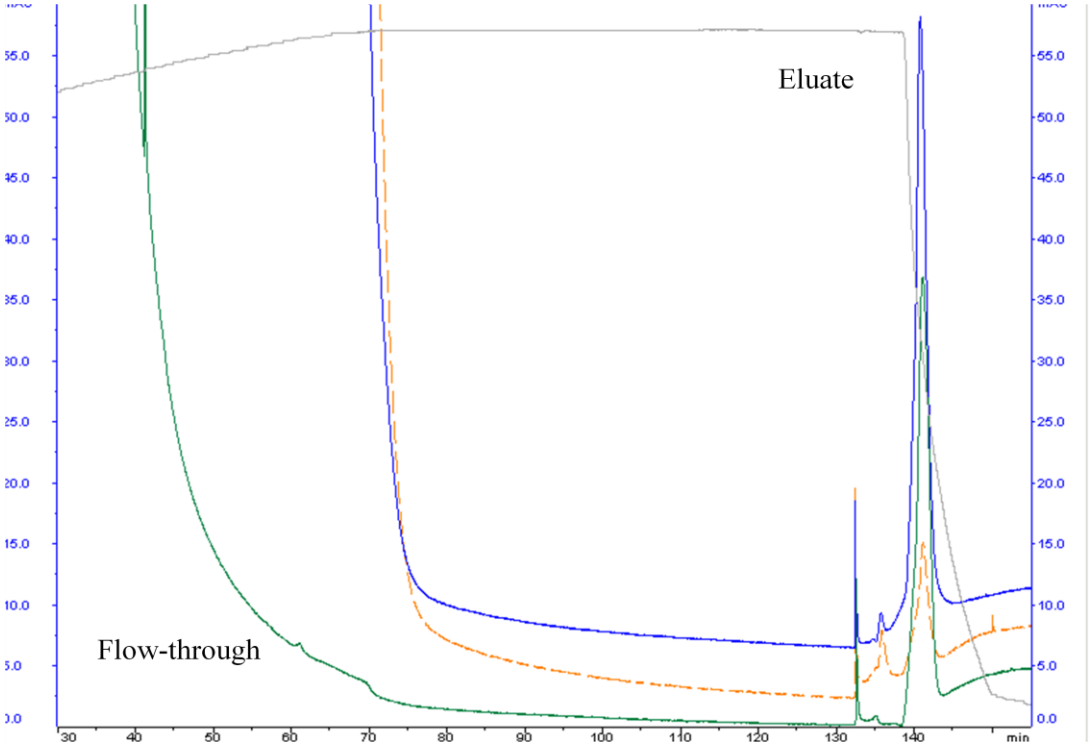

Figure A3.5. Pretreated algae lysate (ammonium sulfate precipitation vs. acidic precipitation) loaded on Capto L resin affinity column. UV_{280}: Blue eluate peak is of ammonium sulfate precipitation; Yellow eluate peak is of no pretreatment; Green eluate peak is of acidic precipitation.

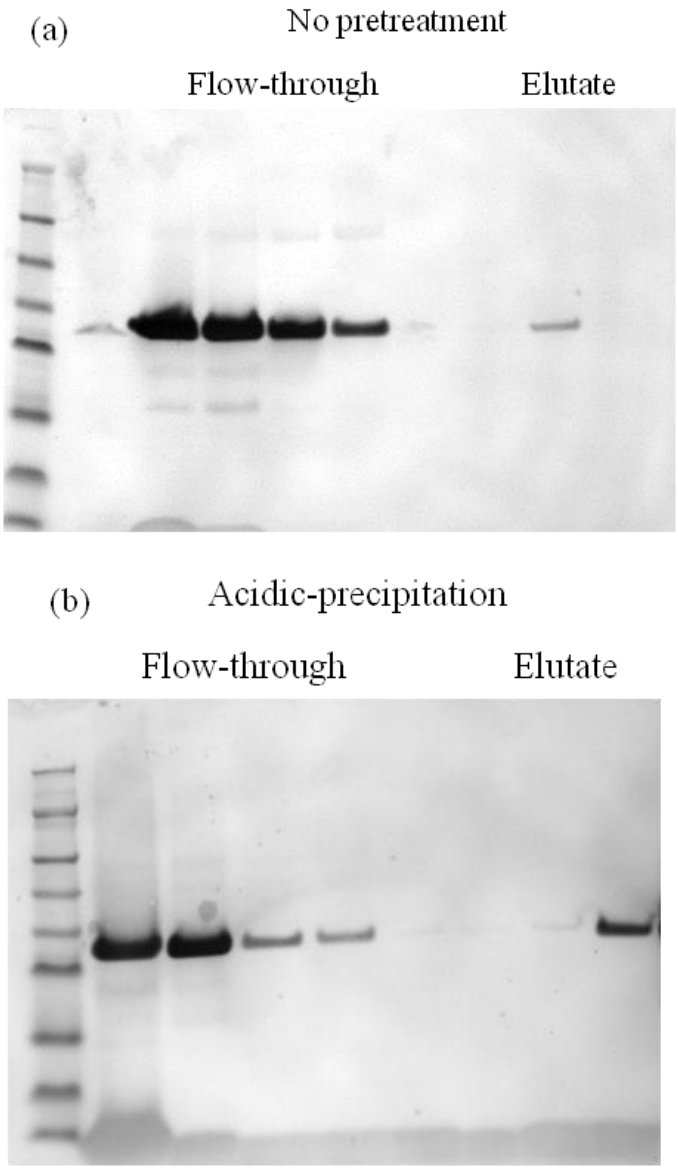

Figure A3.6. Comparison (No pretreatment vs. Acidic precipitation on cell lysate) of Western blot analysis (using anti-FLAG-AP conjugated antibody) of *C. reinhardtii* containing αCD22 scFv purified by Capto L Affinity column (chromatogram in Figure A3.5); (a) No pretreatment; Lane 1: marker; Lane 2 to 7: flow through fractions; Lane 8 to 10: elution fractions. (b) Acidic precipitation on cell lysate; Lane 1: marker; Lane 2 to 7: flow through fractions; Lane 8, 9: elution fractions. There was significant loss of αCD22 scFv in the flow through. Hence Capto L resin affinity chromatography was inefficient for the target protein.